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Analysis and metabolic engineering of lipid-linked oligosaccharides in glycosylation-deficient CHO cells

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Abstract

Glycosylation-deficient Chinese Hamster Ovary (CHO) cell lines can be used to expand our understanding of N-glycosylation pathways and to study Congenital Disorders of Glycosylation, diseases caused by defects in the synthesis of N-glycans. The mammalian N-glycosylation pathway involves the step-wise assembly of sugars onto a dolichol phosphate (P-Dol) carrier, forming a lipid-linked oligosaccharide (LLO), followed by the transfer of the completed oligosaccharide onto the protein of interest. In order to better understand how deficiencies in this pathway affect the availability of the completed LLO donor for use in N-glycosylation, we used a non-radioactive, HPLC-based assay to examine the intermediates in the LLO synthesis pathway for CHO-K1 cells and for three different glycosylation-deficient CHO cell lines. B4-2-1 cells, which have a mutation in the dolichol phosphate-mannose synthase (DPM2) gene, accumulated LLO with the structure Man₅GlcNAc₂-P-P-Dol, while MI8-5 cells, which lack glucosyltransferase I (ALG6) activity, accumulated Man₉GlcNAc₂-P-P-Dol. CHO-K1 and MI5-4 cells both produced primarily the complete LLO, Glc₃Man₉GlcNAc₂-P-P-Dol, though the relative quantity was lower in MI5-4. MI5-4 cells have reduced hexokinase activity which could affect the availability of many of the substrates required for LLO synthesis and, consequently, impair production of the final LLO donor. Increasing hexokinase activity by overexpressing hexokinase II in MI5-4 caused a decrease in the relative quantities of the incomplete LLO intermediates from Man₅GlcNAc₂-PP-Dol through Glc₁Man₉GlcNAc₂-PP-Dol, and an increase in the relative quantity of the final LLO donor, Glc₃Man₉GlcNAc₂-P-P-Dol. This study suggests that metabolic engineering may be a useful strategy for improving LLO availability for use in N-glycosylation.

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Conflict of Interest

None declared.

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Keywords

Chinese Hamster Ovary; Congenital Disorders of Glycosylation; hexokinase; HPLC; N-linked glycan deficiency

Introduction

Glycosylation-deficient Chinese Hamster Ovary (CHO) cell lines offer significant opportunity to further our understanding of the N-glycosylation pathway and the role glycosylation plays in determining structure-function relationships. These mutant cell lines are also useful as model systems for studying Congenital Disorders of Glycosylation (CDG), human diseases caused by defects in the synthesis of N-glycans [1]. The mammalian N-glycosylation pathway involves the step-wise assembly of sugars onto a dolichol phosphate carrier (P-Dol) by specific glycosyltransferases to form a lipid-linked oligosaccharide (LLO) [2,3]. The oligosaccharide can then be transferred from the LLO carrier onto specific asparagine residues within the glycoprotein by an enzyme complex called the oligosaccharide transferase (OST).

LLO biosynthesis begins on the cytoplasmic face of the ER [4] with the transfer of sugar residues from nucleotide-activated sugar donors, such as uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and guanosine diphosphate mannose (GDP-Man), onto P-Dol [5,6]. Two N-acetylglucosamine (GlcNAc) and five mannose (Man) residues are added sequentially by specific glycosyltransferases resulting in an LLO with the structure $\text{Man}_5\text{GlcNAc}_2\text{-P-P-Dol}$ (Fig. 1) [2,5,7,8]. This structure is then flipped across the ER membrane to the luminal face [4], where it is modified by an additional four mannose residues and three glucose (Glc) residues (Fig. 1). The glycosyltransferases on the luminal face of the ER utilize lipid activated monosaccharides, such as mannosylphosphoryl dolichol (Man-P-Dol) and glucosylphosphoryl dolichol (Glc-P-Dol), as sugar donors [2,6]. The final LLO is a dolichol pyrophosphate-linked tetradecasaccharide with the structure $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$ (Fig. 1) [7,9,10].

Over the years, several glycosylation-deficient CHO cell lines have been isolated that are defective in the biosynthesis of N-linked glycans [11,12,13]. B4-2-1 was originally isolated due to a deficiency in mannose 6-phosphate receptor activity [14,15]. Subsequent research showed that the line was unable to synthesize Man-P-Dol [14], presumably due to a defect in dolichol phosphate-mannose synthase (DPM1) activity [16]. Later work pinpointed a mutation in the DPM2 gene in these cells [17,18]. B4-2-1 is part of the Lec15 complementation group, which is characterized by inefficient synthesis of Man-P-Dol, and has been termed Lec15.1 [13,19]. A deficiency in DPM1 is associated with CDG type Ie [20,21].

MI8-5 [22] was derived from the parental CHO-K1 line via a mannose suicide procedure which selected for colonies with low incorporation of mannose into glycoproteins [12]. MI8-5 was shown to lack glucosyltransferase I activity [22]. Glucosyltransferase I (ALG6) is responsible for adding the first glucose residue to the growing LLO on the luminal face of the ER (Fig. 1) [23,24]. A lack of glucosyltransferase I activity is also known to be the cause of CDG type Ic [25].

MI5-4 was also derived via a mannose suicide selection, similar to the MI8-5 mutant [12]. MI5-4 was found to have reduced hexokinase activity, and the levels of GDP-Man were 70% of normal [12]. Hexokinase is responsible for phosphorylating hexoses (6-carbon sugars), such as glucose, fructose, and mannose, into hexose-6-phosphates [26], which are required precursors in the synthesis of nucleotide-activated sugars (Fig. 1). Reduced hexokinase activity, therefore, has the potential to affect the availability of many substrates involved in the

N-glycosylation pathway, such as GDP-Man, UDP-Glc, and UDP-GlcNAc, as well as Man-P-Dol and Glc-P-Dol. Indeed, MI5-4 cells were found to incorporate lower amounts of radiolabeled mannose into their LLOs than the parental CHO-K1 cells [12].

The B4-2-1, MI8-5, and MI5-4 cell lines each have a defect that has been found to impact N-glycosylation. In each case, however, evaluation of the oligosaccharide structures produced by the cells required using techniques that involved pulse-labeling with [2-³H]mannose or [³H]galactose [12,14,22]. Using radioactive pulse-labeling techniques, one cannot determine the steady-state levels of oligosaccharides. Also, detection based on the amount of radiolabeled sugar incorporated into the oligosaccharide could create a bias toward detecting oligosaccharides with a larger number of sugars [27].

In this study, we investigated LLO synthesis in the B4-2-1, MI8-5, and MI5-4 cell lines using a non-radioactive, HPLC-based technique. Unlike pulse labeling, this technique allows for analysis of LLO in cells that are not actively incorporating radioactive precursors, such as those with genetic defects or those grown under adverse cell culture conditions [27]. The steady-state levels of intermediates in the LLO synthesis pathway were analyzed in these glycosylation-deficient cell lines to confirm how certain defects would affect the identity and level of the LLOs available for N-glycosylation. In addition, hexokinase was overexpressed in MI5-4 to determine the affect of increased hexokinase activity on LLO synthesis.

Materials and Methods

Cell culture

Parental CHO-K1 cells were obtained from ATCC and recloned by limiting dilution as described [12]. B4-2-1 [15], MI5-4 [12], and MI8-5 [22] were isolated as described. CHO-K1 cells were maintained in DMEM media (Gibco, #11965) supplemented with 2 mM L-glutamine (Gibco, #25030), 1x Non-essential amino acids (Gibco, #11140), and 10% fetal bovine serum (Gibco, #16140). The other three lines were maintained in α -MEM media (Invitrogen, #41061) with the same supplements. Since the glycosylation-deficient cells are temperature sensitive for growth, all cells were maintained at 34°C in a humidified incubator with 5% CO₂. Cells were passaged using trypsin-EDTA (Gibco, #25300054) and cell counts were determined via hemocytometer.

Expression of hexokinase II

The plasmid containing the hexokinaseII cDNA was obtained from Dr. John E. Wilson at Michigan State University. Briefly, the cDNA for *Rattus norvegicus* hexokinase II (RefSeq ID: NM_012735) was inserted directionally into the pcDNA3 vector (Invitrogen) using the EcoRI and XbaI sites. A BamHI site was added after the cDNA and before the XbaI site via primer design. MI5-4 cells were transfected with pcDNA3-hexokinaseII using Lipofectamine 2000 (Invitrogen, #11668) according to the manufacturer's instructions. After 48 hours, cells were selected with 1000 μ g/ml Geneticin (Invitrogen, # 10131-035) in α -MEM complete media for several days. The transfected pool (MI5-4-hexoII) was then maintained in α -MEM complete media containing 500 μ g/ml Geneticin.

Western blot

Cells were lysed using 1 ml of lysis buffer (25 mM Tris-Cl pH 7.4, 0.5% Triton-X 100, 50 mM NaCl, 2 mM EDTA, 1x Complete protease inhibitors (Roche, #11 836170 001)) per confluent 10 cm dish. Equal amounts of total protein were loaded into each lane of a 10% SDS-PAGE gel then proteins were resolved and transferred to nitrocellulose. The top half of the membrane (75 kDa and above) was blocked with 5% BSA in TBST, then probed with rabbit anti-hexokinase II (Cell Signaling, #2867) followed by HRP-conjugated goat anti-rabbit (Pierce,

#31460). The bottom half of the membrane (50 kDa and below) was blocked with 5% non-fat dry milk in TBST, then probed with anti- β -actin (Abcam, #ab8226) followed by HRP-conjugated rabbit anti-mouse (Sigma, #A9044). Chemiluminescence was detected using the Immun-Star Western C Kit (BioRad, #170 5070) and the BioRad ChemiDoc XRS imager.

LLO analysis

Cells were grown to confluence in 15 cm dishes then 100×10^6 cells were extracted with 20 ml of ice cold methanol. LLOs were extracted and partially purified using the method of Gao and Lehrman [27]. The oligosaccharides that were released from the lipid carriers via acid hydrolysis were purified further by passing the samples through graphitized carbon columns (0.25 ml bed volume) [28]. Oligosaccharides were then labeled with 2-aminopyridine by the Kondo method [29] and analyzed by normal phase HPLC using an Amide-80 column (2×150 mm, $3 \mu\text{m}$, Tosoh Biosciences) with in-line fluorescence detection (300 nm excitation, 360 nm emission). The column was pre-equilibrated with Buffer A (80% acetonitrile (v/v), 20% 10 mM ammonium formate, pH 7.0) and glycans were eluted by two steps of linear gradients of Buffer B (50% acetonitrile, 50% 10 mM ammonium formate, pH 7.0) as follows: 0% Buffer B at 0 min, 50% Buffer B at 12 min, 100% Buffer B at 60 min. Peak identity is determined by comparing the elution position of each oligosaccharide to the elution positions of 2-aminopyridine labeled LLO standards [30,31].

Results and Discussion

LLO distribution in CHO-K1

We first analyzed the LLO produced by the parental CHO-K1 cell line using a non-radioactive, HPLC-based technique. This cell line was investigated first in order to better understand any changes in LLO synthesis that might occur in the CHO glycosylation mutants. LLO from CHO-K1 were isolated and then the oligosaccharides were cleaved from the lipids, fluorescently labeled, and analyzed via normal phase HPLC. The HPLC chromatogram indicated one large peak with an elution time of approximately 54 minutes (Fig. 2.A.). This peak was found to elute at the same position as the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ structure and makes up 77% of the total LLO. Small peaks were also seen at 41, 45, and 48 minutes, corresponding to the elution positions of the $\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ structures respectively.

These results are consistent with previous studies done using $[2\text{-}^3\text{H}]$ mannose radiolabeling techniques, which also indicate that CHO-K1 cells produce primarily $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$ [12,22]. The small amounts of $\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ detected indicate that synthesis of the fully mannosylated LLO ($\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$) proceeds rapidly in CHO-K1, while addition of the glucose residues is slower in these cells, leading to the accumulation of $\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ (See Fig. 1). This result was not detected in prior mannose labeling studies [12]. Accumulation of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$, however, which is the final LLO in the synthesis pathway and the preferred substrate for the OST, suggests that there are no major bottlenecks impeding LLO synthesis in the parental CHO-K1 line.

LLO distribution in B4-2-1

LLO were isolated from B4-2-1 cells and then the oligosaccharides were cleaved from the lipids and fluorescently labeled. When the oligosaccharides were separated and detected via HPLC, one major peak with an elution time of about 24 minutes was seen (Fig. 2.B.). This corresponds to the elution position of the $\text{Man}_5\text{GlcNAc}_2$ oligosaccharide structure. Minor peaks were also seen with elution positions of 27, 32, 35, and 38 minutes, but the vast majority (78%) of the LLO in B4-2-1 has the structure $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$.

Previous research done using [2-³H]-mannose labeling techniques concluded that the B4-2-1 CHO mutant accumulated an LLO that was resistant to endoglycosidase H digestion and smaller than the LLO produced by the parental CHO-K1 cell line [14]. This oligosaccharide was similar in structure to one of the minor species of LLO found in the parental cells [14]. It was suggested that the predominant LLO produced by this cell line was most likely Man₅GlcNAc₂-PP-Dol [14,16]. Definitive determination of the identity of this LLO was not possible, however, because the oligosaccharide structures could only be deduced by comparing their elution positions on gel filtration columns to the elution positions of GlcNAc homopolymers, which were used as standards [14].

Using a non-radioactive HPLC-based LLO analysis technique, we confirmed that the major LLO in B4-2-1 was Man₅GlcNAc₂-PP-Dol. Accumulation of Man₅GlcNAc₂-PP-Dol is reasonable based on the deficiency in DMP2 activity in B4-2-1 cells. DMP2 catalyzes the addition of mannose from GDP-Man onto Dol-P producing the lipid-linked sugar, Man-P-Dol [32]. Man-P-Dol is the required substrate for the luminal mannosyltransferases which catalyze attachment of the final four Man residues onto the LLO core (See Fig. 1) to form Man₆GlcNAc₂-PP-Dol through Man₉GlcNAc₂-PP-Dol [4,33]. Since the required substrate is not available to synthesize the larger LLO, Man₅GlcNAc₂-PP-Dol would be expected to accumulate and become the predominant LLO in B4-2-1 cells.

The availability of the glucosyl donor Glc-P-Dol is unaffected in B4-2-1 cells, which means that Man₅GlcNAc₂-PP-Dol could potentially be glucosylated. Using radioactive labeling experiments, Anand et al. found that approximately 10–30% of Man₅GlcNAc₂-PP-Dol was converted to Glc₃Man₅GlcNAc₂-PP-Dol in Lec15 cells such as B4-2-1 [34]. This indicates that these cells can glucosylate the LLO intermediate Man₅GlcNAc₂-PP-Dol. The low percentage of glucosylated LLO, however, suggests that glucosyltransferase I has a high specificity for its preferred substrate, Man₉GlcNAc₂-PP-Dol. In our LLO analysis of B4-2-1, the minor peaks that eluted after Man₅GlcNAc₂ (Fig. 2.B.) could potentially represent the glucosylated versions of this LLO, Glc₁₋₃Man₅GlcNAc₂-PP-Dol.

LLO distribution in MI8-5

The LLO were also isolated from MI8-5 and analyzed using the HPLC-based LLO analysis technique described here. The chromatogram shows a large peak eluting at about 42 minutes and a smaller peak eluting at 24 minutes (Fig. 2.C.). By comparing these elution positions to those of 2-aminopyridine labeled LLO standards, the smaller peak was identified as Man₅GlcNAc₂, while the larger peak, which represents 70% of the total LLO, was identified as Man₉GlcNAc₂.

Work by Quellhorst et al. indicates that the MI8-5 CHO mutant cannot glucosylate its LLO and that this mutation could be complemented by transfection with the *S. cerevisiae* ALG6 gene [22]. They concluded that the MI8-5 lacked glucosyltransferase I (ALG 6) activity (See Fig. 1) [22]. Our data, obtained using a fluorescent HPLC-based technique, is consistent with the previous work and indicates an accumulation of Man₉GlcNAc₂-PP-Dol in the MI8-5 mutant. This result is reasonable because glucosyltransferase I is responsible for adding the first glucose residue onto the growing LLO. If this first glucose is not present, then there will be no substrate available for ALG8 or ALG 10 and the second and third glucose residues cannot be added. This results in accumulation of the largest possible LLO in MI8-5, Man₉GlcNAc₂-PP-Dol.

LLO distribution in MI5-4

The MI5-4 mutant produced an LLO distribution that was similar to the distribution seen in the parental CHO-K1 line (Fig. 2.A. vs. Fig. 2.D). Indeed, small peaks were seen at 40, 44 and

48 minutes (Fig. 2.D.), corresponding to $\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ respectively. The largest peak (52% of the total LLO) eluted at approximately 53 minutes and corresponds to the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ structure, the preferred substrate for the OST. These peaks were similar to those seen for the parental CHO-K1, however, the HPLC elution profile for MI5-4 also shows a small peak around 24 minutes, corresponding with the $\text{Man}_5\text{GlcNAc}_2$ structure, which was not seen in the CHO-K1 elution profile.

MI5-4 was originally isolated and found to be deficient in hexokinase activity [12]. O'Rear et al. used $[2\text{-}^3\text{H}]$ mannose labeling techniques to analyze the LLO produced by these cells. The labeled oligosaccharides appeared to have the same structure in MI5-4 and CHO-K1 based on their elution profiles on a gel filtration column. The structure of the major LLO was assumed to be $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ based on the elution position of the oligosaccharide compared to GlcNAc homopolymer standards [12].

Similarly, our data indicates that the MI5-4 mutant produces mainly $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, but we also detected low levels of $\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$. The sensitivity of our new technique allows for detection of these minor LLO components which were previously undetectable. It also allows us to precisely identify each intermediate in the LLO synthesis pathway and compare small differences in LLO distribution between cell lines. Indeed, our data indicates that the MI5-4 mutant produced a reduced relative quantity of the final LLO donor, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$, compared to CHO-K1, and increased levels of the incomplete LLOs: $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$, $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$, and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ (Fig. 2.A. vs. Fig 2.D). This observation might be explained by the reduction in hexokinase activity in this cell line. Reduced hexokinase activity would likely diminish the available pools of glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate. Since GDP-Man, UDP-Glc, and UDP-GlcNAc are synthesized from these substrates, and Man-P-Dol and Glc-P-Dol are synthesized from the sugar nucleotides, the levels of these sugar donors are also likely to decrease. In fact, O'Rear et al. did find that the levels of GDP-Man were 30% lower in MI5-4 than in the parental [12]. All of these substrates are required for LLO synthesis and reduced availability could potentially lead to a decrease in the efficiency of each of the glycosyltransferase reactions. This would in turn lead to the buildup of the LLO intermediates at each step in the synthesis pathway and, consequently, a reduction in the amount of the final LLO donor produced.

LLO distribution in MI5-4 overexpressing hexokinase II

In order to examine further whether hexokinase activity may indeed be limiting LLO synthesis in the MI5-4 mutant, hexokinase II was overexpressed in this cell line. The cells were transfected with the gene for *R. norvegicus* (rat) hexokinase II and protein expression was confirmed via western blot (Fig. 3). The antibody used cross-reacts with human and rat hexokinase II (but not Chinese Hamster hexokinase II) so lysate from the human line, 293F, was used as a positive control (lane 5). A band indicating the presence of hexokinase II in the lysate of MI5-4 cells overexpressing hexokinase II (MI5-4-hexoII) was also seen in lane 5. No bands were seen in the negative control lanes containing the lysates of MI5-4 expressing YFP (lane 1), untransfected MI5-4 (lane 3), and MI5-4 expressing hexokinase I (lane 4). Expression of β -actin was used to ensure equal protein loading in all lanes.

The LLO from the MI5-4-hexoII cells were then isolated and analyzed using a fluorescent HPLC-based method and the relative quantities of LLO produced by MI5-4-hexoII were compared to those produced by untransfected MI5-4 cells (Fig. 4). MI5-4-hexoII produced significantly less $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$, $\text{Man}_8\text{GlcNAc}_2\text{-PP-Dol}$, and $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ than MI5-4 and produced lower levels of every LLO from $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ through $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$. Conversely, the MI5-4-hexoII produced significantly more of the

final LLO donor, Glc₃Man₉GlcNAc₂-PP-Dol, than MI5-4. These results suggest that expression of hexokinase II caused an increase in the mannosylation and glucosylation of LLO in the ER. This effect may be due to increased levels of Man-P-Dol and Glc-P-Dol available for use in LLO synthesis resulting from increased hexokinase activity. Increased availability of these sugar donors could increase the efficiency of each step in the LLO synthesis pathway, leading to a relative decrease in the level of each intermediate in the pathway and a relative increase in the level of the final LLO donor. Indeed, this concept is consistent with our data, in which the relative percentage of Glc₃Man₉GlcNAc₂-PP-Dol increased from 47±2.4 % of the total LLO in MI5-4 to 77±2.2% of the total LLO in MI5-4-hexoII.

Conclusions

Cell lines such as B4-2-1, MI8-5, and MI5-4 are extremely useful tools for furthering our understanding of the mammalian N-glycosylation pathway and for determining how specific enzymes affect the identity and quantity of LLOs available for N-glycosylation. In this study, we examined the intermediates in the LLO synthesis pathway for three glycosylation-deficient CHO cell lines using a non-radioactive, HPLC-based LLO analysis method. We found that CHO-K1 and MI5-4 accumulated primarily the fully formed Glc₃Man₉GlcNAc₂-PP-Dol, while MI8-5 and B4-2-1 produced the incomplete intermediates, Man₉GlcNAc₂-PP-Dol and Man₅GlcNAc₂-PP-Dol, respectively. We also found that expression of recombinant hexokinase led to an increase in the relative quantity of Glc₃Man₉GlcNAc₂-PP-Dol produced in MI5-4. Our data implies that the level of hexokinase affects the availability of sugar donors required for LLO synthesis and that reduced hexokinase activity can negatively impact production of the final LLO structure. Overexpression of hexokinase may be an effective strategy for increasing the availability of the final LLO donor for use in N-glycosylation. In the future, glycosylation-deficient CHO cell lines could be used to test various treatments options for CDG.

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Abbreviations

CDG	Congenital Disorders of Glycosylation
P-Dol	dolichol phosphate
LLO	lipid-linked oligosaccharide
Man	Mannose
Glc	Glucose
GlcNAc	N-Acetylglucosamine

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Figure 1. The LLO synthesis pathway

On the cytoplasmic face of the ER, dolichol phosphate is modified by glycosyltransferases, which utilize nucleotide-activated sugar donors, to form $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$. This structure is then flipped across the ER membrane to the luminal face, where additional glycosyltransferases use lipid-activated monosaccharides as sugar donors in order to form the final LLO donor: $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$. The oligosaccharide is then transferred from the dolichol donor onto the polypeptide by the OST enzyme complex. The affected enzymes for B4-2-1 (*DPM2*), MI8-5 (*ALG6*), and MI5-4 (*hexokinase*) are indicated in gray italics.

Figure 2. HPLC profiles of LLO distributions for parental and mutant CHO cell lines
LLO were purified from (A) CHO-K1, (B) B4-2-1, (C) MI8-5, and (D) MI5-4 and then the oligosaccharides were cleaved from the lipids, fluorescently labeled, and detected via HPLC.

Figure 3. Western blot confirming expression of hexokinase II in MI5-4-hexoII
293F cell lysate was used as a positive control, untransfected MI5-4 cell lysate was used as a negative control, and β -actin was used to control for protein loading.

Figure 4. Comparison of relative LLO levels in MI5-4 vs. MI5-4-hexoII

Relative LLO levels (mean $\pm$ standard error, n=3) were quantified using the HPLC data by integrating the area under each peak. M5 = Man₅GlcNac₂, G3M9 = Glc₃Man₉GlcNac₂. Significance: *, p<0.05; **, p<0.005; ***, p<0.001.