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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 $Man_5GlcNAc_2$-P-P-Dol, while MI8-5 cells, which lack glucosyltransferase I (ALG6) activity, accumulated $Man_9GlcNAc_2$-P-P-Dol. CHO-K1 and MI5-4 cells both produced primarily the complete LLO, $Glc_3Man_9GlcNAc_2$-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 $Man_5GlcNAc_2$-PP-Dol through $Glc_1Man_9GlcNAc_2$-PP-Dol, and an increase in the relative quantity of the final LLO donor, $Glc_3Man_9GlcNAc_2$-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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1. 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 $Man_5GlcNAc_2$-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 $Glc_3Man_9GlcNAc_2$-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

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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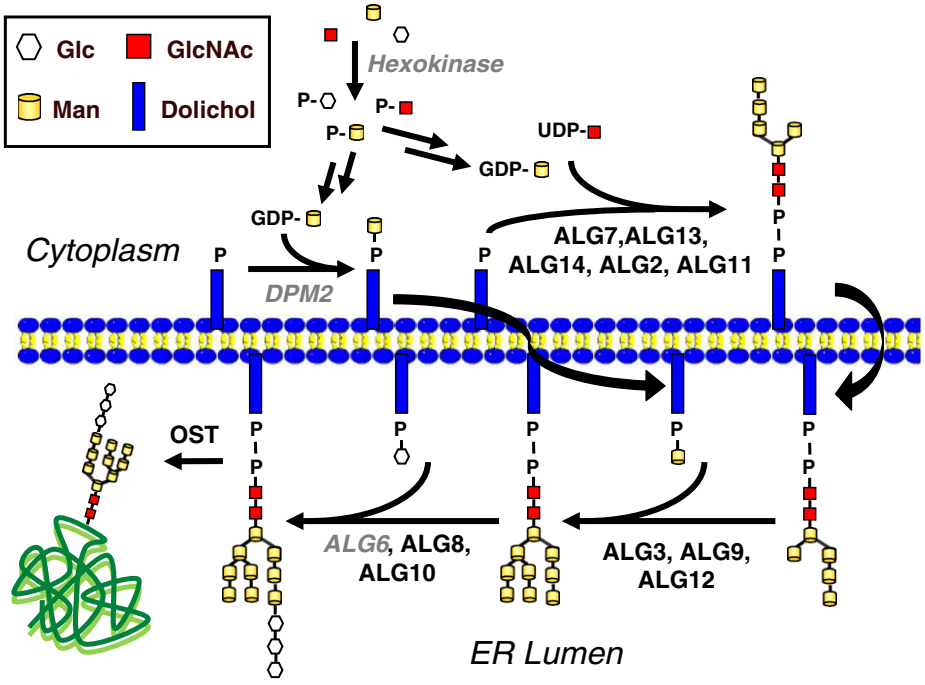

Fig. 1. The LLO synthesis pathway. On the cytoplasmic face of the ER, dolichol phosphate is modified by glycosyltransferases, which utilize nucleotide-activated sugar donors, to form $Man_5GlcNAc_2$-PP-Dol. This structure is then flipped across the ER membrane to the luminal face, where additional glycosyltransferases use lipid-activated monosaccharides as sugar donors in order to form the final LLO donor: $Glc_3Man_9GlcNAc_2$-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.

N-linked glycans [11–13]. 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-^{3}H]mannose or [^{3}H]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 effect of increased hexokinase activity on LLO synthesis.

2. Materials and methods

2.1. 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), 1× 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% CO_2. Cells were passaged using trypsin–EDTA (Gibco, #25300054) and cell counts were determined via hemocytometer.

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