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Fluorophore-assisted carbohydrate electrophoresis: a sensitive and accurate method for the direct analysis of dolichol pyrophosphate-linked oligosaccharides in cell cultures and tissues

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Abstract

Lipid-linked oligosaccharides (LLOs) such as $Glc_3Man_9GlcNAc_2$-P-P-dolichol are the precursors of asparagine (N)-linked glycans, which are essential information carriers in many biological systems, and defects in LLO synthesis cause Type I Congenital Disorders of Glycosylation. Due to the low abundance of LLOs and the limitations of the chemical and physical methods previously used to detect them, almost all studies of LLO synthesis have relied upon metabolic labeling of the oligosaccharides with radioactive sugar precursors such as [^{3}H]mannose or [^{14}C]glucosamine. In this article, a procedure is presented for a facile, accurate, and sensitive non-radioactive method for LLO analysis based on fluorophore-assisted carbohydrate electrophoresis (FACE). First, LLOs are extracted and partially purified. Next, oligosaccharides released from LLOs are labeled with negatively charged fluorophores: 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) or 7-amino-1,3-naphthalenedisulfonic acid (ANDS). A specialized form of polyacrylamide gel electrophoresis is then used to resolve and measure ANTS or ANDS labeled oligosaccharides. Finally, the resolved oligosaccharides are detected and quantified by fluorescence imagers using CCD cameras.

Keywords: Glycosylation; Lipid-linked oligosaccharide; Dolichol; Fluorophore-assisted carbohydrate electrophoresis

1. Introduction

In the lumenal space of the endoplasmic reticulum (ER), asparagine (N)-linked glycoproteins are formed by transfer of preformed oligosaccharide units from lipid-linked oligosaccharide (LLO) donors to nascent polypeptides with asparaginyl residues in the context Asn-X-Ser/Thr [1,2]. Completed LLOs have the structure $Glc_3Man_9GlcNAc_2$-P-P-dolichol, the preferred substrate for oligosaccharyl transferase compared with premature LLO intermediates [3]. LLO synthesis is highly conserved and requires a series of glycosyltransferase reactions. The first seven sugar transfer reactions take place on the cytoplasmic leaflet of the ER membrane and generate the lipid-linked intermediate $Man_5GlcNAc_2$-P-P-dolichol, which requires two equivalents of UDP-GlcNAc and five of GDP-mannose. By a mechanism still not fully understood, $Man_5GlcNAc_2$-P-P-dolichol flips to the lumenal leaflet, where it is the acceptor substrate for seven additional glycosyltransferase reactions using four equivalents of mannose-P-dolichol and three of glucose-P-dolichol as donors. This results in synthesis of $Glc_3Man_9GlcNAc_2$-P-P-dolichol, and this process is defective in the Type I congenital disorders of glycosylation (CDG), a family of at least 12 distinct genetic diseases [4–6]. After the oligosaccharide unit from $Glc_3Man_9GlcNAc_2$-P-P-dolichol is transferred to protein, the resultant N-linked oligosaccharide is processed in the ER by a series of glucosidases and

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mannosidases, in most cases ending in the structure $Man_8GlcNAc_2$. After exiting the ER, N-linked oligosaccharides of glycoproteins undergo additional processing reactions involving glycosidases and glycosyltransferases in the Golgi complex and *trans*-Golgi network. These N-linked oligosaccharides have specific roles in the folding, processing, and export of glycoproteins from the ER, as well as for functions that occur after exiting the secretory pathway [7].

A thorough knowledge of LLO synthesis is therefore essential to understand both ER function and the pathophysiology of diseases like CDG Type I. LLO analysis usually requires hydrolysis of the pyrophosphate bond, allowing the free water-soluble oligosaccharides to be fractionated and characterized, though recently an efficient fractionation of intact LLOs has been reported [8]. Since abundance of LLOs is low, typically of the order of 1 nmol/g tissue [9,10], detection of the oligosaccharides by physical and/or chemical means has been inefficient. It is common to study LLOs that have been made radioactive by incubations of cells, organelle preparations, or tissue slices with appropriate ^3H- or ^{14}C-labeled precursors. The incorporated isotopes then permit facile detection of the oligosaccharides.

Although isotopic approaches have been extremely useful for LLO analysis, they have a number of limitations: (1) Isotopic labeling in normal culture medium is often inefficient unless low glucose concentrations are used (5–10-fold below the physiological range), subjecting the cultures to potential glucose deprivation effects [11]. (2) The results obtained by metabolic labeling may not reflect the true steady-state LLO compositions since they are typically done for a brief incubation period (20–60 min). (3) Due to isotope dilution, it is difficult to determine the actual molar quantity of each LLO species from the amount of radioactivity incorporated. Intermediates with few sugars may be difficult to detect. (4) Metabolic labeling is difficult in living animals. Pool dilution and catabolism would require the use of very large quantities of radioactive compounds. Although LLO compositions from tissue of large animals, like bovine pancreas [9,10], have been studied by direct non-isotopic analysis of the oligosaccharides, these approaches were laborious, required large amounts of tissue, and might not be practical with small experimental animals such as the mouse or rat.

In this review, I describe the use of fluorophore-assisted carbohydrate electrophoresis (FACE) [12,13] to circumvent these problems. With the use of a commercial fluorescence scanner, multiple samples are easily processed. One to two picomoles of oligosaccharide released from each LLO species is detected, and detection is independent of the number of residues per oligosaccharide. In this article, protocols are provided for LLO extraction, partial purification, fluorophore labeling, and quantitation.

2. Methods

2.1. LLO extraction and partial purification

2.1.1. Reagents

CM: chloroform methanol mixed at a ratio of 2:1 (v/v).
CMW: chloroform, methanol, and water mixed at a ratio of 10:10:3 (v/v).
DEAE–cellulose (Bio-Rad, #731-1550), converted to acetate form by washing with 1 M acetic acid (in CMW).
A 3 M NH_4OAc stock solution prepared by dissolving 3 mol NH_4OAc into a total volume of 1000 ml methanol with 3% acetic acid.

2.1.2. Protocol

2.1.2.1. LLO extraction from cultured cells.

1. Wash the cells (90% confluence, $>10^7$ cells, typically four 15 cm dishes for dermal fibroblasts or one 10 cm dish for CHO-K1 cells) with ice cold PBS twice, add room temperature methanol, harvest the cells by scraping, and transfer to a 10 ml glass tube (Kimble, #73790-10).
2. Sonicate for 5–10 min in a waterbath sonicator, and dry under a stream of N_2 gas.
3. Add 10 ml CM to the tube, and sonicate for 5–10 min with occasional vortexing. Centrifuge (3000*g* for 10 min at room temperature) and discard the supernatant. Repeat once.
4. Resuspend pellet in 2 ml methanol by sonication, and dry under N_2 gas.
5. Add 10 ml water to the tube, sonicate for 5–10 min with occasional vortexing, centrifuge, and discard the supernatant. Repeat once.
6. Resuspend pellet in methanol by sonication. Dry under N_2 gas.
7. Add 10 ml CMW to the tube, sonicate 5–10 min with occasional vortexing. Centrifuge and collect the supernatant. LLOs are extracted in this fraction.

2.1.2.2. LLO extraction from animal tissue.

1. Harvested animal tissues should be used immediately or frozen in liquid nitrogen. Fresh or frozen tissues are disrupted into 10 volumes of methanol with a Bronson homogenizer ("polytron") for 30 s at a setting of 6.
2. The methanolic suspension is dried under N_2 gas.
3. The remaining steps are the same as for cultured cells beginning at step 3.

Note. When drying the samples in methanol under nitrogen gas in the first step of LLO extraction, the samples can easily form hardened pellet chucks. To avoid