Teaching dolichol-linked oligosaccharides more tricks with alternatives to metabolic radiolabeling

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The dolichol cycle involves synthesis of the lipid-linked oligosaccharide (LLO) Glc₃Man₉GlcNAc₂-P-P-dolichol (G₃M₉Gn₂-P-P-Dol), transfer of G₃M₉Gn₂ to asparaginyl residues of nascent endoplasmic reticulum (ER) polypeptides by oligosaccharyltransferase (OT), and recycling of the resultant Dol-P-P to Dol-P for new rounds of LLO synthesis. The importance of the dolichol cycle in secretory and membrane protein biosynthesis, ER function, and human genetic disease is now widely accepted. Elucidation of the fundamental properties of the dolichol cycle in intact cells was achieved through the use of radioactive sugar precursors, typically [³H]-labeled or [¹⁴C]-labeled mannose, galactose, or glucosamine. However, difficulties were encountered with cells or tissues not amenable to metabolic labeling, or in experiments influenced by isotope dilution, variable rates of LLO turnover, or special culture conditions required for the use of radioactive sugars. This article will review recently developed alternatives for LLO analysis that do not rely on metabolic labeling with radioactive precursors, and thereby circumvent these problems. New information revealed by these methods with regard to regulation, genetic disorders, and evolution of the dolichol cycle, as well as caveats of radiolabeling techniques, will be discussed.

Key words: dolichol/fluorophore-assisted carbohydrate electrophoresis/lipid-linked oligosaccharide/N-linked glycosylation

With the exception of most protists and some fungi (Samuelson et al. 2005), the lipid-linked oligosaccharide (LLO) Glc₃Man₉GlcNAc₂-P-P-dolichol (G₃M₉Gn₂-P-P-Dol; Figure 1) normally occurs in all eukaryotes. Synthesized as depicted in Figure 2, G₃M₉Gn₂-P-P-Dol is the donor substrate for oligosaccharyltransferase (OT), and is used for N-linked glycosylation of nascent polypeptides as they enter the endoplasmic reticulum (ER) lumen (Wacckter and Lennarz 1976; Hubbard and Ivatt 1981; Kornfeld and Kornfeld 1985).

Advantages and disadvantages of metabolic radiolabeling techniques for the study of dolichol-linked oligosaccharides

Advantages of metabolic radiolabeling
Since 1970s (Spiro et al. 1976; Robbins et al. 1977; Sefton 1977), metabolic radiolabeling has been an essential technique for analysis of LLOs in intact cells [procedures reviewed in Rosner et al. (1982)]. A widely-used approach has been labeling with [³H]mannose, which is metabolized in succession to [³H]mannose-6-P (M₆P), [³H]mannose-1-P (M₁P), GDP-[³H]mannose, and [³H]mannose-P-dolichol (MPD). GDP–mannose is the enzymatic donor for the mannosyl residues of the intermediate M₅Gn₂-P-P-Dol, while MPD is the donor for the remaining mannosyl residues forming M₉Gn₂-P-P-Dol (Figure 2). The presence of multiple mannosyl residues makes this technique very sensitive for detection of G₃M₉Gn₂-P-P-Dol. For example, in the author’s laboratory, a single, 100 mm culture dish containing 5 × 10⁶ Chinese hamster ovary (CHO)-K1 cells can generate sufficient [³H]G₃M₉Gn₂-P-P-Dol for multiple high performance liquid chromatography (HPLC) runs with an automated in-line isotope detector. In a pulse-labeling mode this technique can assess LLO synthesis rates, while continuous labeling for longer periods approaches steady state. Although M₆P is converted by phosphomannose isomerase to fructose-6-P (F₆P), leading to glucose-6-P (G₆P), labeling with [³H]mannose does not extend into these other molecules because isomerization of M₆P to F₆P results in loss of tritium to [³H]H₂O. Another common approach is metabolic radiolabeling with [¹³C]galactose, which is efficiently converted to UDP-[¹³C]glucose and thus can be used to label the glucosyl residues in G₃M₉Gn₂-P-P-Dol. Other strategies for metabolic labeling of LLOs include labeling of GlcNAc residues with radiolabeled glucosamine and labeling the dolichol moiety with radiolabeled acetate or mevalonate.

Disadvantages of metabolic radiolabeling
However useful, metabolic radiolabeling techniques also have limitations: (i) Since LLO pools generally require up to 20 min to turn over (Hubbard and Robbins 1979), much longer labeling periods are needed for steady-state compositional analysis. (ii) Metabolic labeling of LLOs in live animals is challenging because of the difficulty of exposing tissue to sufficiently high concentrations of radioactive precursor. Similar limitations...
apply for LLOs in cells which may not actively incorporate radioactive precursor due to inhibitory culture conditions, genetic defects, the absence of appropriate transport systems, or inefficient metabolic conversion to nucleotide sugar. (iii) The specific activities of the mannosyl residues in LLOs are much lower than that of the starting material due to isotopic dilution with intracellular mannosyl compounds. Therefore, the identities and relative amounts of individual LLO intermediates can be determined, but additional steps must be taken to establish the actual chemical yield of each LLO intermediate. One approach to this problem has been to determine the exact specific activity of the cell-associated GDP–[^3H]mannose, and then apply that value to the radiochemical yield of LLO to calculate the chemical amount (Rush and Waechter 1995). In that study, the caveat of deducing LLO quantity solely from radiochemical yield was demonstrated in a comparison of four different cell lines, where the amounts of LLO labeled with [2–[^3H]mannose in a 60-min period (essentially steady state) varied only 4.8-fold when corrected for the specific activity of GDP–[^3H]mannose, while the uncorrected counts per minute values varied over a 54-fold range. Similar disparities were determined for label in the glycoprotein fractions. This approach was therefore useful for accurately assessing chemical LLO amounts in cultures of fibroblasts from congenital disorders of glycosylation (CDG)-Type I patients labeled with [2–[^3H]mannose (Rush et al. 2000). (iv) LLO analyzes of clinical specimens are laborious and time-consuming because of the requirement for metabolic radiolabeling. For example, diagnosis of LLO defects in Type I CDG patients involves collection of a skin biopsy and establishment of fibroblast cultures, rather than immediate analysis of the specimen. (v) Effects of certain alterations (such as attenuation of protein synthesis) on the LLO pathway are very difficult to study because the alterations may block metabolic labeling (Gao and Lehrman 2002b).

General features of alternatives to metabolic radiolabeling

In theory, the limitations discussed in Disadvantages of metabolic radiolabeling might be circumvented by alternatives that do not require metabolic incorporation of radiolabel. A number of such strategies are listed in Table I. Two of the approaches detect intact LLOs. The other methods detect LLO glycans after release from dolichol-P-P, and involve three steps: (i) chemical modification with a chromophore, fluorophore, or isotope; (ii) chromatographic or electrophoretic separation; and (iii) detection by absorbance, fluorescence, or...
liquid scintillation spectroscopy. However, only a few of these techniques (the final four listed in Table I) appear facile enough for routine laboratory use with large sample numbers, as well as having modest requirements for equipment and operator expertise. The remainder of this article will focus on these alternatives (Figure 3) and review the experiments in which they were used to provide new and important information about LLOs.

**Table I. Alternatives to metabolic radiolabeling of LLO**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mode</th>
<th>Modification</th>
<th>Separation</th>
<th>Detection</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Badet and Jeanloz (1988)</td>
<td>Endo-H cleaved glycans</td>
<td>Reduction with NaBH₄</td>
<td>Amino HPLC</td>
<td>Absorbance at 190 nm</td>
<td>Calf pancreas</td>
</tr>
<tr>
<td>Badet and Jeanloz (1988)</td>
<td>Acid cleaved glycans</td>
<td>Reduction with NaBT₄</td>
<td>Amino HPLC</td>
<td>Scintillation counting</td>
<td>Calf pancreas</td>
</tr>
<tr>
<td>Spiro et al. (1983), Hall et al. (1989)</td>
<td>Acid cleaved glycans</td>
<td>Reduction with NaBT₄</td>
<td>TLC</td>
<td>Autoradiography</td>
<td>Calf thyroid, Ovine NCL tissues</td>
</tr>
<tr>
<td>Hall et al. (1989)</td>
<td>Acid cleaved glycans</td>
<td>Reduction with NaBT₄ and peracetylation</td>
<td>Spherisorb HPLC</td>
<td>Scintillation counting</td>
<td>Ovine NCL tissues</td>
</tr>
<tr>
<td>Faust et al. (1994)</td>
<td>Acid cleaved glycans</td>
<td>Perbenzoylation</td>
<td>Reverse-phase HPLC</td>
<td>Absorbance at 230 nm</td>
<td>Normal and NCL mouse brain</td>
</tr>
<tr>
<td>Gibbs and Coward (1999)</td>
<td>Acid cleaved glycans</td>
<td>None</td>
<td>Biogel P-4</td>
<td>Sugar analysis</td>
<td>Bovine pancreas</td>
</tr>
<tr>
<td>Kelleher et al. (2001)</td>
<td>Intact LLO</td>
<td>None</td>
<td>Amino HPLC</td>
<td>Concanavalin A-HRP conjugate after immobilization in glass tubes</td>
<td>Porcine pancreas</td>
</tr>
<tr>
<td>Kelleher et al. (2001)</td>
<td>Intact LLO</td>
<td>None</td>
<td>Amino HPLC</td>
<td>HPLC after transfer to [¹²⁵Ⅰ]-peptide</td>
<td>Porcine pancreas, yeast</td>
</tr>
<tr>
<td>Gao and Lehrman (2002a)</td>
<td>Acid cleaved glycans</td>
<td>Reduction with ANTS or ANDS</td>
<td>FACE gel</td>
<td>Fluorescence</td>
<td>Dermal fibroblasts, CHO cells, and various mouse tissues</td>
</tr>
<tr>
<td>Grubenmann et al. (2004), O'Reilly et al. (2006)</td>
<td>Acid cleaved glycans</td>
<td>Reduction with 2AB</td>
<td>GlykoSep-N HPLC</td>
<td>Fluorescence</td>
<td>Dermal fibroblasts, yeast</td>
</tr>
</tbody>
</table>

*Discussed in detail in this review; TLC, thin layer chromatography.

Analyses of intact LLOs based upon either transfer of glycan to [¹²⁵Ⅰ]-acceptor peptide with chromatographic fractionation, or solid-state concanavalin A binding with chemiluminescence

**Concanavalin A-binding assay**

Gilmore’s laboratory (Kelleher et al. 2001) developed two general strategies for detection of LLOs. One, a solid-state...
enlarged chemiluminescence (ECL)-based assay, can be used to determine the total amount of LLOs with glycans that bind concanavalin A (Con A) and does not require a source of OT (see below). The ECL-based assay is semiquantitative, and involves immobilizing LLO samples at the bottoms of glass tubes, followed by incubation with a Con A-horseradish peroxidase (HRP) conjugate. The tubes are placed in a rack, and in the dark ECL reagent is added to each tube. The rack is then placed over X-ray film, and after developing the film, the intensity of the exposure under each tube is used to determine the relative amount of LLO in the sample.

**OT assay**

Gilmore’s laboratory (Kelleher et al. 2001) also developed a set of procedures utilizing OT to assess the LLO content of a mixture or an enriched preparation. Notably, a quantitative “endpoint assay” was devised, which typically involves incubation of the LLO sample with a 10 to 100-fold excess of No-Ac-Asn-[125I]Tyr-Thr-NH2 acceptor peptide under conditions with which essentially all of the lipid-linked glycan is transferred. This assay is extremely sensitive because using iodinated peptide with a specific activity of 15,000 cpm/pmol, <0.05 pmol of LLO can be detected after HPLC analysis of [125I]glycopeptides. The yeast OT preparation is preferred over other sources of OT because it has a relatively low discrimination for G3M9Gn2-P-P-Dol over LLO intermediates under the conditions used, and it is also free of interfering glucosidase and mannosidase activities. If assessment of heterogeneity of the LLO preparation is not necessary, total [125I]glycopeptides can be determined by binding to Con A-agarose. Further, if the specific activity of the [125I]acceptor peptide is known, the quantity of each glycopeptide in the preparation can be determined by separation from aminopropyl silica HPLC (giving baseline resolution of glycopeptide products) and liquid scintillation spectroscopy. Since [125I]glycopeptides are purified by binding to Con A-agarose before HPLC, glycopeptides with M2Gn2 to G3M9Gn2 can be detected.

**Development of a LLO purification procedure**

Modification of a diethylaminoethyl-cellulose approach (Spiro et al. 1979) allowed development of a preparative HPLC procedure for obtaining individual enriched LLOs. The ECL and endpoint methods were used to characterize the LLO fractions obtained (Kelleher et al. 2001). For example, a highly enriched (90–95%) preparation of G3M9Gn2-P-P-Dol was obtained from pancreas, with G3M9Gn3-P-P-Dol and G3M9Gn2-P-P-Dol accounting for the remaining LLOs in the preparation. This purification of pancreatic LLOs was an improvement over earlier methods that appeared subject to degradation by glycosidases during isolation. The usefulness of these techniques was also demonstrated by isolation of LLO intermediates from a Saccharomyces cerevisiae mutant strain, Δalg3. The major LLO purified, as expected, was M2Gn2-P-P-Dol. Interestingly, the assay identified appreciable quantities of G3M9Gn2-P-P-Dol (Figure 4A), which was not detected by previous [2-3H]mannose labeling of alg3 strains, possibly due to the superior sensitivity of the endpoint assay.

The mechanism, subunit heterogeneity, and substrate specificity of OT

The preparative LLO isolation, coupled with the detection methods discussed above, were instrumental for studies on the catalytic mechanism of OT. However, rather than rely upon endpoint techniques, these experiments were not designed to deplete the LLO substrate, and in some cases tested competition between LLO. In a detailed kinetic analysis (Karaoghlu et al. 2001), the specificity of yeast OT for G3M9Gn2-P-P-Dol over M2Gn3-P-P-Dol was investigated, using highly purified preparations of each LLO and [125I]-acceptor peptide. Distinct catalytic and regulatory (activator) binding sites for LLOs were identified, with the conclusion that binding of either LLO to the regulatory site enhances occupancy of G3M9Gn2-P-P-Dol of the catalytic site. This helped explain why, in isolation, both LLOs were equally good donors, but when mixed in a 1:1 ratio, G3M9Gn2-P-P-Dol was a 5-fold better donor than M2Gn3-P-P-Dol. It is also consistent with observations that glycoproteins from mutant yeast strains and animal cells with a homogenous supply of an LLO intermediate are not as extensively hypoglycosylated as might be anticipated if OT was selective for G3M9Gn2-P-P-Dol under all conditions. The very existence of such a mechanism suggests that under normal conditions, LLO pools in some cells may not be predominantly G3M9Gn2-P-P-Dol, but instead may contain G3M9Gn3-P-P-Dol mixed with a high proportion of LLO intermediates.

Preparation of highly enriched LLOs was also important for establishing the distinct donor substrate specificities of isoforms of mammalian OT, an octameric enzyme (Figure 4B) (Kelleher and Gilmore 2006). Specifically, isoforms containing the STT3-A subunit were relatively less active, though more selective for G3M9Gn2-P-P-Dol, compared with OT isoforms with the STT3-B subunit (Kelleher et al. 2003). To demonstrate this, OT preparations from canine pancreatic microsomes were resolved by high-performance ion-exchange (Mono-Q) chromatography into fractions containing either the STT3-A or STT3-B subunits, and in which the other STT3 subunit was undetectable. The enzymatic properties of these OTs were then determined with highly purified LLO preparations. Compared with the STT3-A form of OT, the STT3-B form of OT had 5–6-fold higher turnover numbers with G3M9Gn2-P-P-Dol, and 17–20-fold higher turnover numbers with M2Gn3-P-P-Dol. The Km value for acceptor tripeptide was independent of the donor LLO for the STT3-B enzyme. However, the STT3-A enzyme had a higher affinity for acceptor than the STT3-B enzyme when G3M9Gn2-P-P-Dol was the donor, and a lower affinity when M2Gn3-P-P-Dol was the donor, explaining in large part the differences in turnover number. The use of purified LLOs allowed the demonstration of two-site (catalytic and activator) kinetics for the mammalian enzyme as had been identified with the yeast enzyme discussed above, and the observation that the Vmax of the STT3-B enzyme was 8–12-fold higher than that of the STT3-A enzyme. To assess selectivity, mixtures of M2Gn3-P-P-Dol and G3M9Gn2-P-P-Dol (10:1 ratio) were incubated with each enzyme in competition assays to quantify glycopeptide products and determine a donor preference ratio. Remarkably, G3M9Gn2-peptides were enriched only 2.6-fold (relative to the 10:1 starting mixture) for the STT3-B enzyme, while the
donor preference ratio for the STT3-A enzyme was 14.7-fold (Figure 4B). Both were broadly expressed, but some tissue differences were identified (for example, STT3-A was higher in liver and pancreas, tissues with high secretory capacity). Both were found in human dermal fibroblasts, with the STT3-B form accounting for 70% of OT. The abundance of the less-selective form of OT may help explain the observation that, in dermal fibroblasts, OT failed to discriminate significantly between the LLO intermediates M3-5Gn2-P-P-Dol and G3M9Gn2-P-P-Dol (Gao et al. 2005).

**Verification of CDG-Ij**

Both the endpoint assay and the preparation of purified G3M6Gn2-P-P-Dol were used to verify a deficiency of UDP–GlcNAc:Dolichol-P GlcNAc-1-P transferase (GPT) in CDG Type Ij (Wu et al. 2003). The expected phenotype of a GPT deficiency is a loss of G3M6Gn2-P-P-Dol, no abnormal accumulation of LLO intermediates, and catalytically normal OT. Initial experiments using [2-3H]mannose labeling of LLOs suggested that CDG-Ij fibroblasts contained less G3M6Gn2-P-P-Dol than normal, but as discussed above interpretation of this type of result was highly dependent upon the intracellular specific activity of the metabolic label. Using pure exogenously added G3M6Gn2-P-P-Dol, OT in microsomes from a CDG-Ij patient’s dermal fibroblasts has the same activity as normal OT. However, when assayed with only endogenous LLO, CDG-Ij OT activity in the endpoint assay was reduced by half. This confirmed that the G3M6Gn2-P-P-Dol supply was deficient in the patient’s fibroblasts.

**Analysis of LLO pools in genetically diverse unicellular eukaryotes**

Sequencing the genomes of certain protists (unicellular eukaryotes) has suggested, due to the absence of specific LLO synthesis genes, that these organisms would not synthesize G3M6Gn2-P-P-Dol (Samuelson et al. 2005). Accumulation of smaller LLOs was predicted: M5Gn2-P-P-Dol for *Trichomonas vaginalis* and *Entamoeba histolytica*, and M9Gn2-P-P-Dol for *Cryptococcus neoformans*. These predictions were borne out by the endpoint assay (Figure 4C). In these studies the endpoint assays used total cellular membranes in the absence of detergent as an endogenous source of OT, and detected transfer of glycans from endogenous LLOs to exogenously added [125I]tripeptide. The results were confirmed by analyzes of the same organisms by [2-3H]mannose labeling and Bio-Gel P-4 chromatography, with detection of the expected [3H]glycans.

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**Fig. 4.** Multiple applications of LLO analysis by OT assays. (A) Identification of G3M7Gn2 in *S. cerevisiae*Δalg3 LLOs in an endpoint assay (Kelleher et al. 2001, reproduced and modified with permission, copyright 2001 Oxford University Press). Positions of tripeptide (NYT)-conjugated products are indicated. GN on figures labels denotes GlcNAc. (B) Determination of the selectivity for G3M6Gn2-P-P-Dol over M6Gn2-P-P-Dol in a 1:10 mixture of the SST3-A and SST3-B forms of OT in a competition assay (Kelleher et al. 2003, reproduced and modified with permission, copyright 2003 Elsevier Press). (C) Atypical LLOs in three protists (Samuelson et al. 2005, reproduced and modified with permission, copyright 2005 National Academy of Sciences, USA).
Analyzes of free LLO glycans by conjugation of fluorophores to exposed reducing termini

Overview of fluorescence-based LLO detection

Two independent techniques have been used to label free LLO glycans at their reducing termini with fluorophores, followed by chromatographic or electrophoretic separation, and then fluorescence detection. Thus, both methods involve release of the glycan (exposing the reducing terminus) from the lipid carrier, and require the prior removal of lipid conjugates that are not related to dolichol pyrophosphate, and/or methods of glycan release that are selective for dolichol pyrophosphate-linked glycans. The endpoint assay discussed above is not subject to this requirement because it only detects lipid-linked saccharides that are true OT substrates, i.e., linked to dolichol-P-P. However, lipid-linked saccharides from cells in which the dolichol moiety is defective and therefore hinders OT reactivity [for example, cells of the lec9 genotype deficient in polyprenol reductase (Rosenwald and Krag 1990)] might be better studied by fluorescent methods or the solid-state assay discussed above, rather than the endpoint assay.

Identification of CDG-Ik by labeling LLO glycans with 2-aminobenzamide

LLO glycans were modified by reductive amination with the fluorophore 2-aminobenzamide (2AB), and fractionated by HPLC with a GlykoSep-N column (Prozyme, Inc., San Leandro, CA; Grubenmann et al. 2004). This technique was used to screen CDG-I cell cultures for LLO synthesis mutations that might affect a step preceding the addition of mannosyl residues, in which case a loss of G_{3}M_{0}G_{12}-P-P-Dol might be evident by [2-3H]mannose labeling, but the associated abnormal LLO would not be detected. 2AB-labeled LLO glycans from normal fibroblasts were identified as G_{3}M_{0}G_{12}-2AB, which was almost undetectable in fibroblasts from an unassigned CDG patient. In contrast, samples from the patient’s fibroblasts contained abundant quantities of G_{3}G_{2}-2AB. Judging from fluorescence yields, the amount of G_{3}G_{2}-P-Dol from the patient’s cells appeared similar to the amount of G_{3}M_{0}G_{12}-P-P-Dol in normal cells (Figure 5A). This would be expected for a metabolic defect that interfered with addition of the first mannol residue. This was confirmed by demonstrating that both Alg1 alleles in the patient were abnormal, leading to a new CDG classification, Type Ik.

2AB analysis of LLOs to determine enzyme activities

Labeling of LLO glycans with 2AB was also used to determine the GDP–mannose dependent activities and specificities of the purified recombinant Alg2 and Alg11 LLO transferases (Figure 5B) (O’Reilly et al. 2006). Beginning with chemoenzymatically-synthesized Man_{3}GlcNAc_{2}-P-P-Dol, Alg2 was convincingly shown to generate the Man_{3}GlcNAc_{2}-P-P-Dol biosynthetic intermediate in vitro. The LLO glycans of the Alg2 products were released from pyrophosphate linkage to dolichol, coupled to 2AB, and characterized by HPLC (GlykoSepN with fluorescence detection; Prozyme, Inc.) in conjunction with diagnostic glycosidic digestions. The Man_{3}GlcNAc_{2}-P-P-Dol made by this approach was also used as substrate for reactions with Alg11. By labeling product LLO
glycans with 2AB, Alg11 was shown to synthesize the Man3GlcNAc2-P-P-Dol biosynthetic intermediate. In conclusion, this study demonstrated that Alg2 and Alg11 each contribute two mannosyl residues during Glc3Man9GlcNAc2-P-P-Dol synthesis. Since these four mannosyl residues were the last to be assigned to a specific transferase, the enzymes responsible for each sugar residue in Glc3Man9GlcNAc2-P-P-Dol are now known.

**Characterization of LLO glycans by FACE**

The general strategies underlying fluorophore-assisted carbohydrate electrophoresis (FACE) have been reviewed (Jackson 1996; Starr et al. 1996). FACE fluorophores bear amino groups for coupling to reducing termini of saccharides by reductive amination. For oligosaccharides with at least three sugars, separation and detection are achieved with a “profiling” gel after coupling to the fluorophores 7-amino-1,3-naphthalenedisulfonic acid (ANDS) or 8-amino-naphthalene-1,3,6-trisulfonate (ANTS). ANDS and ANTS carry, respectively, two and three anionic sulfate modifications. ANDS may be preferred, due to a higher fluorescence yield. For monosaccharides and disaccharides, separation and detection are accomplished with borate-impregnated “monosaccharide composition” gels after coupling to 2-aminoacridone (AMAC), which lacks sulfate. Thus, AMAC should be used for GlcNAc1,2-P-P-Dol, and ANDS or ANTS with Glc0,3Man1,9GlcNAc2-P-P-Dol. In all cases, high percentage polyacrylamide gels in a “mini-gel” format are used with high voltage and cooling. The number of samples run on each gel is determined by the choice of comb used to form wells. Since special low-ultraviolet (UV) absorbing glass plates are used, the fluorescent compounds are detected under UV light without disassembly of gel sandwich, allowing a series of images to be acquired during the electrophoresis. Sensitivity with a fluorescence scanner equipped with a charged-coupled devise (CCD) camera is in the range of 1–2 pmol, while simple transilluminators (of the type used to visualize ethidium bromide-stained deoxyribonucleic acid gels) and UV viewing chambers can be used with lower sensitivity. Baseline resolution of oligosaccharides containing up to 14 sugars, and in some cases oligosaccharides with the same sugar composition but different isomeric configuration, can be achieved. Methods and equipment for FACE detection of LLO glycans from cultured cells and tissues have been described (Gao and Lehrman 2002a; 2003, 2006). Validation of the technique included chemical and enzymatic assessments of the fluorophore-conjugated LLO glycans, detection of glycan intermediates from cell lines with well-characterized defects in the LLO pathway, incorporation of [2-3H]mannose label into conjugated glycans, and loss of conjugated glycans by treatment with tunicamycin. Control experiments failed to reveal significant artifactual degradation during isolation of mouse tissue LLO glycans.

**FACE analysis of LLO heterogeneity in normal mouse tissues, and of LLO accumulation in mouse models of neuronal ceroid lipofuscinosis (Batten disease)**

Prior to introduction of the FACE technique, data on LLO compositions in normal animal tissues were highly limited, and dealt mainly with pancreas (Table I). Since most mouse tissues, as determined by FACE, have roughly 1 nmol LLO/g, FACE-LLO analysis with small fresh or frozen mouse tissue samples is highly feasible (Gao and Lehrman 2002a). FACE showed that G3M9Gn2-P-P-Dol was the most abundant LLO species in each mouse tissue, reaffirming that the general principles for LLO synthesis discerned from cell culture studies also applied to tissues. However, in kidney and spleen LLO intermediates were relatively abundant, and in aggregate accounted for most of the LLO pool. The total LLO content varied over a ten-fold range, being highest in kidney and lowest in testis and lung.

FACE was useful for reevaluating and extending earlier reports of LLO accumulation in tissues (especially brain) of animals and patients characterized by neuronal ceroid lipofuscinosis (NCL), or Batten disease (reviewed in Cho et al. 2005). The NCLs are neurodegenerative lysosomal storage diseases associated with accumulation of autofluorescent storage material of uncertain composition. There are eight genetically distinguishable NCL subtypes. The recent acquisition of multiple mouse models of NCL by gene disruption or spontaneous mutation made it feasible to systematically test the accumulation of LLOs in freshly isolated NCL brain, compared with age-matched controls (Cho et al. 2005). FACE demonstrated accumulation of LLOs (typically M4,9Gn2-P-P-Dol) in the brains (as well as kidney and liver) of protein palmitoyltransferase 1-deficient (PPTT1−/−; Figure 6A) and four other mouse models of NCL, but not in three mouse models of nonNCL lysosomal storage disorders. Further, the accumulated mouse NCL brain LLOs had structures consistent with a catabolic pathway because endoglycosidase H-sensitive isomers of M4,9Gn2-P-P-Dol were detected, while NCL microsomes exhibited no apparent LLO biosynthetic defect. Importantly, brain NCL membranes were a mixture of microsomes of normal density containing normal LLOs with endogenous OT activity, and heavy membranes carrying autofluorescent material as well as the accumulated (degraded) LLOs, without apparent endogenous OT activity. One scenario proposed was failure of the autophagosome or another organelle to completely degrade microsomal-derived LLOs.

**FACE analyzes of LLO turnover: effects of translation attenuation and ER stress**

Synthesis of G3M9Gn2-P-P-Dol from radiolabeled sugar precursors is inhibited by agents that block either transcription (Hubbard and Robbins 1980) or translation (Spiro et al. 1976; Schmitt and Elbein 1979; Grant and Lennarz 1983). As reviewed (Gao and Lehrman 2002b), several models were proposed, but elucidation of the mechanism was hampered by the inability to follow the fate of G3M9Gn2-P-P-Dol, precisely because it could no longer be detected by radiolabeling. To address this problem, analysis of the effects of the translation inhibitors cycloheximide and puromycin on LLO synthesis in CHO-K1 cells was performed by metabolic labeling with [2-3H]mannose in tandem with FACE. These experiments showed that although translation inhibitors prevented incorporation of radiolabel, they had no effect on the quality or quantity of LLOs detected by FACE, indicating greatly slowed LLO turnover caused by the absence of polypeptide acceptor substrate for OT (Gao and Lehrman 2002b). Translation inhibition also reduced synthesis of [3H]lipids formed in Dol-P-dependent in vitro reactions with UDP-[3H]GlcNAc, UDP-[3H]glucose, and GDP-[3H]mannose. This was due to trapping of Dol-P as G3M9Gn2-P-P-Dol by
translation inhibitors; consequently, Dol-P was no longer replenished by recycling of Dol-P-P. Both sets of results strongly supported the proposal for a limited pool of Dol-P (Hubbard and Robbins 1980). Reduction of in vitro synthesis of the [3H]-lipids was detected only with cells that were gently permeabilized with streptolysin-O, not with microsomal membranes. This result suggested that the physical perturbation required to prepare microsomal membrane mixed a
functional primary Dol-P pool (the limited pool) with a secondary pool that normally was not involved in LLO synthesis, and explained why previous studies using microsomal membranes from cells pretreated with translation inhibitors failed to detect loss of Dol-P.

Translation attenuation is a physiological process, and of particular interest here is PKR-like ER kinase (PERK) (Harding et al. 1999). PERK is an ER-associated Type I transmembrane kinase with a luminal domain which senses ER stress (such as that caused by excessive protein misfolding), and consequently drives transautophosphorylation (activation) of PERK’s cytoplasmic kinase domain. The substrate of PERK-P is eukaryotic initiation factor (eIF) 2α, which participates in translation initiation. Because eIF2α-P interferes with translation initiation, activation of PERK (as well as other eIF2α kinases) causes translation attenuation. An important consequence is a reduction in the load of client polypeptide, alleviating ER stress. This translation attenuation also affects G3M9Gn2-P-P-Dol pools (Shang et al. 2007). Using the approach described above, translation attenuation by activated PERK reduced turnover of G3M9Gn2-P-P-Dol, with extensive loss of [3H]G3M9Gn2-P-P-Dol synthesis but no effect on G3M9Gn2-P-P-Dol detected by FACE (Figure 6B). However, the significance of reducing turnover of G3M9Gn2-P-P-Dol during a response to ER stress was not clear. Accumulation of LLO intermediates such as M2,5Gn2-P-Dol is a likely cause of aberrant N-glycosylation, leading to protein unfolding defects and activation of PERK. Therefore, the effects of ER stress-dependent attenuation of translation on accumulated LLO intermediates were examined. Surprisingly, even mild activation of PERK (20–35% attenuation of translation) strongly promoted extension of M2,5Gn2-P-P-Dol intermediates to G3M9Gn2-P-P-Dol, and resulted in modification of nascent polypeptides with G3M9Gn2 rather than truncated glycans. Thus, the combined uses of metabolic radiolabeling and FACE to assess the LLO pool in ER-stressed cells demonstrated that PERK can counteract LLO synthesis dysfunction by slowing LLO turnover.

Unexpected complexity from PMM deficiency in CDG-Ia: caveats of low-glucose media

CDG-Ia results from mutations in the PMM2 gene encoding phosphomannomutase (PMM). PMM converts M6P (formed by the action of hexokinase upon mannose, or by the action of phosphomannose isomerase upon F6P) to M1P. M1P is condensed with guanosine triphosphate by pyrophosphorylase to GDP–mannose, with pyrophosphate as a byproduct. As discussed earlier, GDP–mannose supplies five mannosyl residues to G3M9Gn2-P-P-Dol directly, and four via MPD (Figure 2). Several studies reported G3M9Gn2-P-P-Dol biosynthesis defects in CDG-Ia dermal fibroblasts, as well as deficient protein N-linked glycosylation, reviewed in Gao et al. (2005). These would be expected consequences of PMM deficiency. Paradoxically, other studies examining specific glycoproteins in CDG-Ia fibroblasts found no N-linked glycosylation deficiency. Studies reporting LLO biosynthesis and/or N-glycosylation defects used metabolic radiolabeling approaches, while studies reporting no N-glycosylation defect used antibody methods. A consistent difference between these two sets of studies is that the former used media with low glucose (0–0.5 mM) to enhance metabolic labeling, while the latter set used conventional media with physiological glucose. No metabolic labeling studies of CDG-Ia LLOs in conventional media had been reported, presumably because the incorporation of radioactivity in LLOs would have been too low for reliable detection.

FACE showed that G3M9Gn2-P-P-Dol was efficiently synthesized in CDG-Ia fibroblasts with physiological glucose (Figure 6C), and that LLO profiles for CDG-Ia fibroblasts were essentially indistinguishable from normal LLO profiles (Gao et al. 2005). At 2.5 mM glucose (just below physiological levels), control fibroblast LLOs lacked significant abnormality by FACE, while CDG-Ia profiles showed strong accumulation of M5Gn2-P-Dol. With 0.5 mM glucose, FACE analysis showed that M5Gn2-P-Dol accumulated and the total amount of LLO was greatly diminished in both cell types. These results led to two important conclusions. First, cultured CDG-Ia fibroblasts did not faithfully reproduce the patients’ glycosylation defects. It remains to be determined whether decreased production of M1P is sufficient to account for the glycosylation deficiency in patients’ hepatocytes, or whether an additional consequence of PMM deficiency may also play a part. Second, the use of low-glucose media should be used with caution. In addition to dermal fibroblasts, unintended effects of such conditions have now been documented by FACE analysis with other cell types (Shang et al. 2007). Specifically, 20–30 min culture of CHO-K1 cells and mouse embryonic fibroblasts with 0.5 mM glucose did not qualitatively alter the LLO profile, i.e. G3M9Gn2-P-P-Dol was still the predominant LLO. However, for both cell types there was a significant quantitative decrease of G3M9Gn2-P-P-Dol.

Stimulation of G3M9Gn2-P-P-Dol discharge by M6P

Since PMM deficiency in CDG-Ia might be expected to increase M6P, the effect of M6P on LLO synthesis was examined in vitro by both FACE (with CHO-K1 cells and hepatocytes) and labeling with GDP–[3H]mannose (with dermal fibroblasts) (Gao et al. 2005). Both techniques yielded the same unexpected result: M6P (but neither M1P, G6P, G1P, nor F6P) caused discharge of G3M9Gn2-P-P-Dol (but no other LLOs) from the ER membrane, with appearance of free G3M9Gn2. Stoichiometric FACE measurements showed a several-fold excess of free G3M9Gn2 over G3M9Gn2-P-P-Dol generated during a 1-h incubation, indicating that Dol-P-P released by the action of M6P on G3M9Gn2-P-P-Dol was recycled to Dol-P for additional rounds of G3M9Gn2-P-P-Dol synthesis. This raised the possibility that the glycosylation defect of CDG-Ia patients might be due to a synergistic effect, with M6P causing excessive discharge of G3M9Gn2-P-P-Dol, and replacement by new G3M9Gn2-P-P-Dol synthesis being hindered due to a deficient supply of M1P for synthesis of GDP–mannose.

Future perspectives

For most investigators, metabolic labeling of LLOs with radioactive sugar precursors should remain the primary approach. However, the alternatives reviewed above can be used to complement radiolabeling. Novel applications of these alternatives are anticipated for the near future. These include analyses of tissues in CDG-I animal models, as well as animal models.
for neuronal storage disorders, muscular dystrophies, metabolic syndrome, and other diseases potentially involving LLOs; diagnosis of CDG-I patient material immediately after biopsy; analyzes of LLOs in extracts of pathogenic microbes that are too hazardous to safely transport or that do not incorporate radiolabel; and testing of novel hypotheses requiring facile quantitative LLO measurements. The ability to transport and analyze LLOs in unlabeled extracts should facilitate collaborative studies because regulatory hurdles regarding the exchange of live cultures can be avoided.

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Conflict of interest statement

None declared.

Abbreviations

AMAC, 2-aminoacidone; ANDS, 7-amino-1,3-naphthalenedisulfonic acid; ANTS, 8-amino-naphthalene-1,3,6-trisulfonate; CCD, charged-coupled devise; CDG, congenital disorders of glycosylation; CHO, Chinese hamster ovary; Con A, concanavalin A; DNA, deoxyribonucleic acid; ECL, enhanced chemiluminescence; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; F6P, fructose-6-P; Glc3Man9GlcNAc2-P-P-dolichol; Glc6P, glucose-6-P; GPT, GlcNAc-1-P transferase; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; LLO, lipid-linked oligosaccharide; M1P, mannose-1-P; M6P, mannose-6-P; MPD, mannose-P-dolichol, NCL, neuronal ceroid lipofuscinosis; OT, oligosaccharyltransferase; PERK, PKR-like ER kinase; PMM, phosphomannomutase; PPT1, protein palmitoyltransferase 1; TLC, thin layer chromatography; UV, ultraviolet.

Note added in proof

Readers are encouraged to refer to Spiro et al. (1983), the earliest of the entries in Table I, which discusses many of the key points covered here in Advantages and disadvantages of metabolic radiolabeling techniques.

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