Coupling Of The Dolichol-P-P-Oligosaccharide Pathway To
Translation By Perturbation-Sensitive Regulation Of The Initiating
Enzyme, GlcNAc-1-P Transferase

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Running Title: Inhibition Of LLO Initiation By Translation Arrest
Summary

In mammalian cells, inhibition of translation interferes with synthesis of the lipid-linked oligosaccharide (LLO) Glc3Man9GlcNAc2-P-P-dolichol as measured with radioactive sugar precursors. Conflicting hypotheses have been proposed, and the fundamental basis for this regulation has remained elusive. Here, fluorophore-assisted carbohydrate electrophoresis (FACE) was used to measure LLO concentrations directly in cells treated with translation blockers. Further, LLO biosynthetic enzymes were assayed in vitro with endogenous acceptor substrates using either cells gently permeabilized with streptolysin-O (SLO) or microsomes from homogenized cells.

In CHO-K1 cells treated with translation blockers, FACE did not detect changes in concentrations of Glc3Man9GlcNAc2-P-P-dolichol or early LLO intermediates. These results do not support earlier proposals for feedback repression of LLO initiation by accumulated Glc3Man9GlcNAc2-P-P-dolichol, or inhibition of a GDP-mannose dependent transferase. With microsomes from cells treated with translation blockers there was no interference with LLO initiation by GlcNAc-1-P transferase (GPT), mannose-P-dolichol synthase, glucose-P-dolichol synthase, or LLO synthesis in vitro, as reported previously. Surprisingly, inhibition of all of these was detected with the SLO in vitro system. Additional experiments with the SLO system showed that the three transferases shared a limited pool of dolichol-P that was trapped as Glc3Man9GlcNAc2-P-P-dolichol by translation arrest. Overexpression of GPT was unable to reverse the effects of translation arrest on LLO initiation, and experiments with FACE and the SLO system showed that overexpressed GPT was not functional in vivo although it was highly active in microsomal assays.
Thus, the combined use of the SLO in vitro system and FACE showed that LLO biosynthesis depends upon a limited primary pool of dolichol-P. Physical perturbation associated with microsome preparation appears to make available a secondary pool of dolichol-P, masking inhibition by translation arrest, as well as activating a non-functional fraction of GPT. The implications of these results for the organization of the LLO pathway are discussed.
Introduction

In eukaryotes, the lipid-linked oligosaccharide (LLO) Glc3Man9GlcNAc2-P-P-dolichol serves as the donor of oligosaccharide units that are transferred by oligosaccharyltransferase (OT) to appropriate asparaginyl residues in nascent polypeptides within the lumen of the endoplasmic reticulum (ER), forming glycoproteins with asparagine-linked (N-linked) Glc3Man9GlcNAc2 glycans (1). The pathway for LLO synthesis has been elucidated by a combination of biochemical and genetic methods, and to date mutations in seven genes essential for LLO synthesis have been identified as the causes of Congenital Disorders of Glycosylation (CDG) Types Ia-g (2,3). LLO synthesis is initiated by the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol-P by a specific tunicamycin (TN)-sensitive GlcNAc-1-P transferase (GPT) (4). This reaction occurs on the cytoplasmic face of the ER membrane (5). GlcNAc-P-P-dolichol is then extended to Man5GlcNAc2-P-P-dolichol, a key LLO intermediate, by a series of cytoplasmically-oriented reactions that catalyze the transfer of one residue of GlcNAc from UDP-GlcNAc and five residues of mannose from GDP-mannose. Cytoplasmically-oriented Man5GlcNAc2-P-P-dolichol then flips to the lumenal leaflet (6,7) in a process involving the Rft1 protein (8). This lumenally-oriented LLO then becomes an acceptor for the transfer of four additional mannose residues from mannose-P-dolichol (MPD) and three glucose residues from glucose-P-dolichol (GPD), generating Glc3Man9GlcNAc2-P-P-dolichol. MPD and GPD are synthesized by the transfer of mannose or glucose from GDP-mannose or UDP-glucose, respectively, to cytoplasmically oriented dolichol-P, and then must flip to the lumenal leaflet to participate in LLO synthesis. Thus, along with the 14 specific LLO transferases, two synthases are needed for MPD and GPD, and flippases are needed.
for MPD, GPD, and Man$_3$GlcNAc$_2$-P-P-dolichol. In animals, transferases that use MPD and GPD as donor substrates have an additional requirement for the product of the Lec35 gene (9).

It is now clear that the LLO pathway in mammalian cells is subject to a number of regulatory features that presumably ensure an adequate supply of the completed LLO Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol for glycoprotein synthesis. For example, in a reciprocal manner GPT and MPD synthase (MPDS) each appear to be positively regulated by the product of the other (10). Extension of LLO intermediates to Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol is stimulated by ER stress, which itself can be a result inadequate supplies of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (11,12). LLO synthesis is dependent upon the amount of dolichol-P (7), which can be elevated in cells by increasing cis-isoprenyltransferase activity (13,14). Taken together, control mechanisms such as these reflect the great importance of synthesis of adequate amounts of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol in ER quality control (15).

Approximately two decades ago, several laboratories determined that new synthesis of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol in mammalian cells incubated with radioactive sugars ceased after the addition of inhibitors of mRNA or protein synthesis ((16-19), reviewed in (4)). At least three hypotheses were proposed to explain this coupling of LLO synthesis with translation. Hubbard and Robbins suggested that in the absence of a polypeptide acceptor, due to "limited availability of the carrier lipid", all dolichol-P available for LLO initiation was trapped as Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (16). Since MPD synthesis did not appear to be inhibited by translation inhibitors (17,18), it was further suggested that the "rate limiting pool might be a fraction of the total dolichol-P" and thus be reserved for synthesis of GlcNAc-P-P-dolichol. However, there was no direct evidence for separate pools of dolichol-P in vivo. Subsequently, the idea that translation blockers reduced the supply of dolichol-P available for LLO initiation was questioned when it was shown that dolichol-P
added to the culture medium stimulated Glc₃Man₉GlcNAc₂-P-P-dolichol synthesis in untreated cells, but not in cells treated with translation inhibitors (18,20).

A second hypothesis, proposed by the Elbein group, suggested that the absence of polypeptide acceptors caused Glc₃Man₉GlcNAc₂-P-P-dolichol to accumulate, and that Glc₃Man₉GlcNAc₂-P-P-dolichol could then act as a feedback inhibitor of GPT (17,20). Two observations were particularly relevant. First, in translation-inhibited cells some loss of GlcNAc-P-P-dolichol synthesis was detected by incubation with [³H]glucosamine. Second, LLO synthesis was partially restored by addition of a cell-permeant acceptor peptide for OT intended to discharge a portion of the accumulated Glc₃Man₉GlcNAc₂-P-P-dolichol. However, the hypothesized accumulation of total cellular Glc₃Man₉GlcNAc₂-P-P-dolichol was not confirmed by direct chemical measurement, and introduction of translation inhibitors after addition of [³H]sugar precursors did not appear to significantly increase the amounts of [³H]Glc₃Man₉GlcNAc₂-P-P-dolichol. Further, inhibition was detected in mutants that produced Man₅GlcNAc₂-P-P-dolichol instead of Glc₃Man₉GlcNAc₂-P-P-dolichol.

Apart from these hypotheses, Grant and Lennarz proposed that inhibition of translation resulted in elevation of cytoplasmic GTP which acted as an inhibitor of cytoplasmically-oriented GDP-mannose dependent LLO transferases. This proposal was supported by their findings that in vivo synthesis of early LLO intermediates up to Man₁GlcNAc₂-P-P-dolichol was not blocked by translation inhibitors (18). Since it was shown previously that a step prior to Man₅GlcNAc₂-P-P-dolichol synthesis was inhibited (16), the hypothesis predicted the accumulation of one or more intermediates, presumably Man₂₋₄GlcNAc₂-P-P-dolichol. However, no accumulated intermediates were identified.
Unfortunately, the radiolabeling methods used with intact cells to formulate these hypotheses raised several complications. For example, the ability to monitor early LLO intermediates was limited since, compared with Glc₃Man₉GlcNAc₂-P-P-dolichol, these molecules contain fewer sugar residues that can be labeled. These types of problems prevented accurate measurements of the actual amounts of various LLOs. Further, because only labeled LLOs could be assessed, the fates of unlabeled pre-existing LLOs were not known.

In this study the control of LLO synthesis by translation was re-examined by employing the recently developed fluorophore-assisted carbohydrate electrophoresis (FACE) approach for direct measurement of LLO compositions in intact cells (21). Moreover, measurements of LLO biosynthetic activities in vitro were made either with cells gently permeabilized with streptolysin-O (SLO), or with microsomes prepared by conventional homogenization techniques. Our results show that translation blockers interfere with LLO synthesis by trapping the dolichol-P available for LLO initiation by GPT as Glc₃Man₉GlcNAc₂-P-P-dolichol, and reveal that the function and regulation of GPT, MPDS, and GPD synthase (GPDS) require properties of the ER that are retained in SLO-permeabilized cells but lost in microsomal preparations. The results directly support the hypothesis of Hubbard and Robbins for a limited pool of dolichol-P that is restricted for LLO synthesis. Upon re-evaluation many of the apparently contradictory results from other studies were found to be consistent with this hypothesis, and differences in experimental conditions appear to account for remaining discrepancies.
Experimental Procedures

Cell Culture-- Culture media and supplies were from Life Technologies/Gibco, except bovine sera (Atlanta Biologicals). CHO-K1 cells, the LLO biosynthesis defective mutants Lec15.2 and Lec35.1 (9), and Tn-10, a CHO-K1 transfectant stably overexpressing GPT (22) were cultured as described previously in Ham's F-12 medium containing 20 mM Na-HEPES, pH 7.2, 2% fetal bovine serum, and 8% calf serum in a humidified atmosphere with 5% carbon dioxide at 37 degrees until 80%-90% of confluence.

Incubation of cells with radioactive metabolic precursors--[^H]mannose: Cells were incubated in medium with 0.5 mM glucose (i.e., 10% of glucose in complete medium) in the presence of 50 µCi/ml [2-[^H]] mannose (23 Ci/mmol; Amersham) for 20 min at 37 degrees, rinsed twice with ice-cold PBS, and then scraped and sonicated with methanol to initiate LLO extraction (9,21).

[^S]methionine: Cell were incubated in complete medium supplemented with 20 µCi/ml[^S]-L-methionine (Amersham Pharmacia Biotech) for 20 min. as described (23). Whole cell lysates were prepared in RIPA buffer (150 mM NaCl, 1.0% (w/v) Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-Cl (pH 8.0), 1 mM phenylmethlysulfonylfluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, all from Sigma) and resolved by SDS-polyacrylamide gel electrophoresis. The total protein radioactivity in each lane was measured with a phosphorimager (Fuji).

Analysis of LLOs-- Intact cells, permeabilized cells, and microsomes were sonicated with an excess of methanol. The suspensions were dried under N₂ gas, and sequentially extracted with
chloroform-methanol (2:1) (CM), pure water, and chloroform-methanol water (10:10:3) (CMW) (24). For HPLC analysis of $[^3]$Hmannonse-labeled LLOs, the material in the CMW extract was treated with weak acid to generate soluble oligosaccharides, and then reduced with NaBH$_4$ (25). FACE analyses of unlabeled LLOs were performed as described (21). Briefly, oligosaccharide profiling gels (Glyko) were used to analyze oligosaccharides modified with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS). Monosaccharide composition gels were prepared (N.G., unpublished) similar to those offered by Glyko, but with less interfering background material in the region used to measure chitobiose, and used for saccharides modified with 2-aminoacridone (AMAC). The gel was imaged with a Biorad Fluor-S MultiImager using a 530DF60 filter. Electronic gel images were generated, and individual fluorescent species were quantified with Quantity One software supplied with the scanner.

**In vitro systems-- Microsomal system:** Cells were swollen and homogenized, and microsomes were recovered by centrifugation as described (26). Approximately 80 µg microsomal membrane protein was recovered per 10$^7$ cells. **Streptolysin-O (SLO) permeabilization:** Cells were treated on wet ice with SLO (Murex brand, distributed by Corgenix, United Kingdom; supplied as a lyophilized powder containing PBS and reconstituted with ice-cold water), then incubated with 37 degree transport buffer to allow SLO pores to form, exactly as described (27). Experiments directly comparing results with both in vitro systems used the same cell equivalents.

**In vitro assays for GPT activity and LLO synthesis--** Transport buffer (27) was used in 2 ml assays with both microsomes and SLO-cells. Nucleotide sugar donors were supplied exogenously, but only endogenous dolichol-P was used. For GPT, unless indicated otherwise, assays included 0.1
μCi/ml of UDP-[³H]GlcNAc and were performed at 37 degrees for 30 min. Lipid products from microsomal reactions were recovered by organic extraction as described (28), and those from SLO-cells were recovered by extraction of cells with chloroform-methanol (2:1) and back-washing with chloroform-methanol-water (3:48:47). MPDS and GPDS were assayed by the same procedure, except that UDP-[³H]GlcNAc was replaced by 0.1 μCi/ml GDP-[³H]mannose and 0.1 μM UDP-[³H]glucose, respectively. For LLO synthesis, assays included 1 μM UDP-GlcNAc and 0.2 μCi/ml of GDP-[2-³H]mannose and were performed at 37 degrees for 10 min, at which point 1 μM GDP-mannose was added and the assay continued (chase) for another 5 min. LLOs were recovered by extraction into chloroform-methanol-water (10:10:3) as described (29).

**Oligosaccharyltransferase acceptor peptides**-- Both the acceptor peptide Ac-Asn-Tyr-Thr-CONH₂, described earlier (20), and the non-acceptor peptide Ac-Gln-Tyr-Thr-CONH₂ were obtained by custom synthesis (Synpep Corp.). The supplier reported that the purity of each peptide assessed by HPLC was at least 97%, and the syntheses were confirmed by mass spectroscopy. Peptides were dissolved in pure water.
Results

Experimental model—To aid the reader, Figure 1 presents a model supported by this study for control of the LLO biosynthetic cycle by protein synthesis. The cycle is initiated by transfer of GlcNAc-1-P to dolichol-P (Dol-P) by GlcNAc-1-P transferase (GPT). After extension of GlcNAc-P-P-dolichol to Glc₃Man₉GlcNAc₂-P-P-dolichol and transfer of oligosaccharide to nascent polypeptide by oligosaccharyl transferase (OT), the resultant dolichol-P-P is converted to dolichol-P by the CWH8 pyrophosphatase (30) for another round of LLO synthesis. The model has three key features. (i) Transfer of oligosaccharide to protein is the rate-limiting step in the cycle. At steady state under normal conditions, most of the dolichol-P available for LLO synthesis is in the form Glc₃Man₉GlcNAc₂-P-P-dolichol. (ii) In the absence of protein synthesis, initiation by GPT is inhibited because it requires a limited primary pool of dolichol-P acceptor that becomes trapped as Glc₃Man₉GlcNAc₂-P-P-dolichol. (iii) Inhibition of LLO initiation is relieved by perturbation of cells and preparation of microsomes. Thus, perturbation causes intermingling of the limited primary pool of dolichol-P with a secondary pool that does not normally have access to GPT. However, this intermingling is prevented by gentle permeabilization with the pore-forming toxin streptolysin-O (SLO).

Use of FACE to test the effects of translation inhibitors on LLO synthesis—As expected from prior studies (16), treatments of CHO-K1 cells with cycloheximide or puromycin for 1 hour inhibited protein synthesis by 99% (Figure 1, panel A) and incorporation of [³H]mannose into LLO (CMW 10:10:3 extract) by 98% (panel B). When total LLO compositions from similarly treated cells were determined by FACE (panels C and D) no LLO accumulation, from GlcNAc-P-P-
dolichol to Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol, was detected. Therefore, these data do not support hypotheses that propose interference with one or more LLO mannosyltransferases (which would cause accumulation of LLO intermediates) (18) or feedback inhibition of LLO initiation by accumulated Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (17,20). Note that GlcNAc$_{1,2}$-P-P-dolichol appeared to be barely detectable by FACE (no more than 1 pmol/10$^7$ cells), and was therefore at least 20-40 fold less abundant than Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (20-40 pmol/10$^7$ cells).

To further test the possible role of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol as a feedback inhibitor, Lec15.2 and Lec35.1 cells were examined since these accumulate Man$_5$GlcNAc$_2$-P-P-dolichol and produce no detectable Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol. Lec35 cells, in contrast with Lec15, do not glucosylate their Man$_5$GlcNAc$_2$-P-P-dolichol (9). FACE did not detect any net LLO accumulation in Lec15 or Lec35 cells treated with translation blockers. These mutant lines were both sensitive to the effects of translation inhibitors on LLO synthesis detected by metabolic labeling (data not shown) as reported earlier (20). Thus, there is no evidence from these experiments that accumulation of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol, or any other glucosylated LLO, is necessary for inhibition of LLO synthesis.

**Assay of GPT Activity In SLO-permeabilized cells reveals attenuation by translation inhibitors**-- Consistent with earlier reports (17,18), we detected no effects for GPT (Figure 3), MPDS, GPDS (see below), and [${}^{3}$H]-LLO synthesis (not shown) in microsomal membranes prepared from CHO-K1 cells treated with translation inhibitors. In the course of these studies, it was noticed that the inhibition of LLO labeling with [${}^{3}$H]mannose in adherent cells was partially relieved by detachment of the cells from culture dishes just prior to labeling. Thus, we considered the possibility that cellular perturbation might counteract the effect of translation inhibition on a key
step in the dolichol pathway, in a manner highly reminiscent of the effects of perturbation on cells lacking the Lec35 gene product (9, 29). Although Lec35p has an essential role in reactions requiring MPD or GPD in intact cells, the glycosylation defective phenotype in Lec35 mutants is lost by various forms of physical perturbation including preparation of microsomes. Gentle permeabilization of the plasma membrane of Lec35 cells with SLO does not affect the Lec35 phenotype, however, permitting the analysis of the Lec35 defect under in vitro conditions (9).

As shown in Figure 3 (panel A), while treatment of CHO-K1 cells with 0.2 mM cycloheximide had no effect on GPT activity measured in microsomes with endogenous dolichol-P acceptors, GPT activity was inhibited by 80 - 90% when assayed in SLO-permeabilized cells. This difference was consistently observed over a range of UDP-GlcNAc concentrations (0.84-54 nM) and incubation times (5-40 minutes; data not shown), and the in vitro activities were highly sensitive to a specific inhibitor of GPT, tunicamycin (TN; panel A). Comparable results with CHO-K1 cells were obtained with 0.4 mM puromycin, and similar effects of translation blockers on in vitro GPT activity were obtained with Lec15 (panel B) and Lec35 cells (not shown). GPT was not directly inhibited by 0.2 mM cycloheximide or 0.4 mM puromycin because neither affected GPT activity when added to SLO-permeabilized CHO-K1 cells (data not shown).

These results have two important implications. First, conventional microsomal preparations do not faithfully reflect the factors that govern GPT activity (see Discussion), presumably by making a secondary pool of dolichol-P available. Such factors are preserved by permeabilization of cells with SLO, possibly because this treatment subjects the cells to minimal physical perturbation. Even scraping the SLO-permabilized cells from dishes increased the remaining GPT activity resulting from translation arrest by approximately 50% (data not shown). Second, in cells that are not continuously synthesizing protein, GPT activity measured after permeabilization with SLO is
highly reduced in a manner that is consistent with the loss of $[^3\text{H}]-\text{LLO}$ synthesis in vivo. The residual GPT activity (10-20\%) may be due to minor perturbation that could not be controlled during permeabilization with SLO.

*LLO synthesis is diminished in cells treated with translation blockers and permeabilized with SLO*-- If the diminished GPT activity in SLO-permeabilized cells represents a preservation of the effects of translation inhibitors on LLO synthesis in vivo, LLO synthesis in vitro with permeabilized cells should be similarly affected. Cells were treated with cycloheximide for various periods up to 1 hour, permeabilized, and assayed either for GPT activity with UDP-$[^3\text{H}]\text{GlcNAc}$, or total LLO synthesis with a mixture of UDP-GlcNAc and GDP-$[^3\text{H}]\text{mannose}$. In the latter case, the reactions included a chase with unlabeled GDP-mannose to extend partially mannosylated LLO intermediates. As shown in Figure 4 (panel A), GPT activity and LLO synthesis declined steadily during the 1 hour series of treatments with cycloheximide. As discussed above, assays performed with microsomes from similarly treated cells did not reveal significant losses of either activity. Loss of LLO synthesis lagged behind the loss of GPT activity. One possible factor is that $[^3\text{H}]\text{mannose}$ would still be incorporated into pre-existing LLO intermediates even though initiation was blocked. The existence of such pre-existing chains acting as $[^3\text{H}]\text{mannose}$ acceptors was verified by performing similar incubations in the absence of UDP-GlcNAc and in the presence of tunicamycin (data not shown). HPLC analyses (panel B) of the LLO chains indicated by panel A revealed predominantly Man$_9$GlcNAc$_2$-P-P-dolichol, demonstrating that MPD was available in the SLO system after translation arrest.
Exogenously expressed GPT has no apparent functional activity in SLO-treated cells and does not compensate for effects of translation inhibitors—If translation inhibition affected LLO initiation by GPT, overexpression of GPT might compensate. Tn-10 cells are CHO-K1 cells stably transfected with a cDNA encoding hamster GPT. They have 30-40 fold greater GPT mRNA and resistance to TN than parental cells, and approximately 10-fold higher enzyme activity in microsomal assays ((22) and Figure 5, panel A). Surprisingly, SLO-treated Tn-10 cells had GPT activities that were equivalent to those in SLO-treated parental cells and parental microsomes (Figure 3). The similarities of GPT activities in SLO-treated normal and Tn-10 cells were consistent, with linear increases in product formation with a variety of UDP-[3H]GlcNAc concentrations (1-72 nM) and incubation periods (5-30 minutes). Thus, the activity of exogenously expressed GPT is essentially silent in assays with SLO-treated cells, although it is readily detected in microsomal assays. This conclusion was supported further by the finding (Figure 5, panel B) that, like parental cells, cycloheximide treatment of Tn-10 cells diminished both GPT activity and LLO synthesis after permeabilization with SLO.

GPT expression does not correspond to LLO quantities synthesized in cells—The LLO pool in Tn-10 cells differs qualitatively from that in CHO-K1 cells in that Tn-10 cells accumulate truncated LLO, mostly Man₅GlcNAc₂-P-P-dolichol with some Man₀GlcNAc₂-P-P-dolichol, and contain relatively little Glc₃Man₉GlcNAc₂-P-P-dolichol (22). Similar results were obtained in cells in which GPT activity was increased by gene amplification (31). The possible basis for this property of Tn-10 cells is considered in the Discussion. CHO-K1 and Tn-10 cells produce similar amounts of total [3H]-LLO when assessed by labeling intact cells with [3H]mannose (22). Since Tn-10 cells overexpress GPT, the enzyme that initiates LLO synthesis, Tn-10 cells would have been
expected to contain considerably more $[^3\text{H}]-\text{LLO}$. The absence of a substantial quantitative
difference could be explained if the overexpressed GPT failed to participate in LLO synthesis \textit{in vivo}, as it fails to participate in GlcNAc-P-P-dolichol synthesis \textit{in vitro} with the SLO system.

FACE was therefore used to directly measure the amounts LLO in Tn-10 cells. As shown
in Figure 6, panel A, the total amount of Man$_5$GlcNAc$_2$-P-P-dolichol in Tn-10 cells was
approximately double the Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol found in CHO-K1 cells. Interestingly,
this modest increase appears to be a result of the LLO extension defect rather than overexpression
of GPT, since two CHO-K1 mutants that also accumulate Man$_5$GlcNAc$_2$-P-P-dolichol, Lec15 (not
shown) and Lec35 (panel A), had similar LLO increases relative to CHO-K1 cells. By labeling the
dolichol moieties of LLOs by incubation with $[^3\text{H}]$mevalonate, a similar correlation was observed
with 3E11 cells overexpressing GPT and B421 cells lacking MPDS in that each had approximately
1.5 times the amount of LLO in parental CHO-K1 cells (32). GlcNAc$_{1,2}$-P-P-dolichol measured by
FACE was undetectable in Tn-10 cells (no greater than 1 pmol/10$^7$ cells) in the absence or presence
of translation inhibitors (not shown).

Since the overexpression of GPT apparently resulted in functionally silent enzyme in intact
cells, it was reasoned that this overexpression should not alter the effects of translation inhibitors on
LLO synthesis. As shown in Figure 6, cycloheximide inhibited the synthesis of
$[^3\text{H}]\text{Man}_{5,9}\text{GlcNAc}_{2}$-P-P-dolichol in Tn-10 cells as efficiently as it inhibited synthesis of
$[^3\text{H}]\text{Glc}_3\text{Man}_9\text{GlcNAc}_{2}$-P-P-dolichol in the parental CHO-K1 cells (panel B), without affecting the
total Tn-10 Man$_{5,9}$GlcNAc$_{2}$-P-P-dolichol detected by FACE (panel A).

The differences in the abilities of the SLO system and the microsomal system to detect the
effects of GPT overexpression, and of translation inhibition, were multiplicative. With the \textit{in vitro}
conditions used in this study, comparable equivalents of control CHO-K1 microsomes and SLO-
treated CHO-K1 cells gave similar GPT activities (Figure 3). By contrast, the GPT activity in microsomes from Tn-10 cells treated with cycloheximide was approximately 35-40 times greater than the GPT activity of the same cells permeabilized with SLO (Figure 5, panel A).

Translation arrest depletes dolichol-P used by MPDS and GPDS--Since the UDP-GlcNAc donor substrate was supplied exogenously for SLO-permeabilized cells, and the catalytic site of GPT is located at the cytoplasmic face of the ER membrane (5), it was unlikely that the decrease of GPT activity caused by translation inhibition was due to an effect on the supply of UDP-GlcNAc. Examination of water-soluble $[^3]$H]-labeled products by ion-exchange gave no indication of enhanced degradation of UDP-GlcNAc by translation-arrested cells (data not shown). Conversely, since both the dolichol-P and GPT in the SLO system are endogenous, effects on these components seemed more plausible.

If the inhibition of GPT activity after cycloheximide treatment was due to reduced availability of dolichol-P, we reasoned that other dolichol-P dependent reactions might also be inhibited. As shown in Table I, both MPDS and GPDS activities were unaffected by cycloheximide treatment if assayed in a microsomal system, but inhibited when assayed in SLO-treated cells. This result ruled out specific inhibition of GPT, and the restoration of activity in microsomal assays demonstrated that MPDS and GPDS, like GPT, used limited primary dolichol-P pools.

GPT and MPDS compete for the same limited pool of dolichol-P--The experiment presented in Table I raised the question of whether GPT and MPDS shared the same limited pool of dolichol-P, or used separate limited pools. Evidence for shared pools in vivo was provided earlier.
by analyses of $[^3]H$mevalonate labeled dolichol conjugates in cells genetically or pharmacologically modified to increased or decrease GPT and MPDS activities (32). Enzymatic evidence for shared pools was reported in two earlier studies (33,34) but these used microsomal systems. To establish directly whether these two enzyme systems shared the same limited pool of dolichol-P, competition experiments were performed with cells permeabilized with SLO but not treated with translation blockers.

In preliminary experiments with nucleotide $[^3]H$-sugars of similar concentration and specific activity, the MPDS reaction in SLO-treated CHO-K1 cells transferred 5 to 10 times more sugar to endogenous acceptor than the GPT reaction. Thus, if these enzymes shared the same pool, it was expected that MPDS would consume a greater proportion of dolichol-P and compete more strongly than GPT. Pre-incubation of SLO-treated cells with 10 mM UDP-GlcNAc had no effect on MPDS activity, in the absence or presence of TN (data not shown). However, as shown in Figure 7 (upper panel), pre-incubation with 1 mM GDP-mannose before addition of UDP-$[^3]H$GlcNAc (group 3) inhibited GPT activity by about 80% compared with incubations in the absence of GDP-mannose (group 1). Inhibition was greatly reduced (approximately 20%) if the GDP-mannose was added after UDP-$[^3]H$GlcNAc (group 2), even though the GDP-mannose was able to drive a considerable portion of the pre-formed $[^3]H$GlcNAc-P-P-dolichol into LLO.

Inhibition of GPT was negligible if Lec15.2 cells were used (lower panel), demonstrating that inhibition of GPT in permeabilized CHO-K1 cells by GDP-mannose required synthesis of MPD. In all cases only small quantities of the $[^3]H$GlcNAc label were recovered in the protein fraction.

*Addition of oligosaccharyltransferase acceptor peptide to cycloheximide-treated cells reverses inhibition of LLO synthesis in vivo and inhibition of GPT activity in vitro*—If the inhibitory effects
of cycloheximide on LLO synthesis and GPT activity are due to trapping of the limited pool of dolichol-P as Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol, inhibition should be prevented by inclusion of an appropriate peptide acceptor for oligosaccharyltransferase. Such a peptide should discharge the Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol and generate a pool of dolichol-P-P to be recycled to dolichol-P (see Figure 1). As listed in Table II inclusion of the peptide Ac-Asn-Tyr-Thr-CONH$_2$, an acceptor for oligosaccharyltransferase, lessened the inhibitory effects of cycloheximide while a non-acceptor control peptide, Ac-Gln-Tyr-Thr-CONH$_2$, had no appreciable effect. The net effect of 500 µM acceptor peptide was restoration of approximately 9% of control LLO synthesis, suggesting that while the effect of the peptide was specific, it discharged only a fraction of the Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol that is normally used during protein synthesis.

This level of improvement of LLO synthesis with acceptor peptide is consistent with the much stronger effect on GPT activity (approximately 250% of untreated controls). GlcNAc$_{1,2}$-P-P-dolichol was not detected by FACE after incubation of $10^7$ untreated SLO-permeabilized cells with saturating (10 mM) UDP-GlcNAc (data not shown). Since FACE is sensitive enough to detect 1-2 pmol of GlcNAc$_{1,2}$, and FACE analyses of the same cells detected 20-40 pmol Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (discussed above), in untreated cells the ratio of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol to dolichol-P in the limited pool seems to be at least 20:1. In cycloheximide-treated cells, a discharge of 9% of the LLO pool in vivo should therefore increase the limited pool of dolichol-P by at least 180%. This would account for the observed effect on GPT activity in vitro.

*The activities of GPT, MPDS, and GPDS in vitro are restored by addition of acceptor peptide to cycloheximide-treated SLO-permeabilized cells*--The experiments discussed to this point demonstrate that translation arrest inhibits GPT, MPDS, and GPDS (Table I); that GPT and total
LLO synthesis depend upon a limited pool of dolichol-P that can be trapped as Glc₃Man₉GlcNAc₂-P-P-dolichol (Table II); and that at least two of the enzymes, GPT and MPDS, share the same limited pool of dolichol-P (Figure 7). To test these conclusions jointly, acceptor and non-acceptor peptides were added directly to SLO-permeabilized cells, both with and without prior treatments with cycloheximide, and each enzyme activity was measured. In all experiments (Figure 8) the non-acceptor (control) peptide was virtually without effect (compare groups 1 and 2, 4 and 5). However, the acceptor peptide markedly increased the activity of each enzyme in cycloheximide-treated cells (group 6). This indicates that all three enzymes share a limited pool of dolichol-P that can be trapped as Glc₃Man₉GlcNAc₂-P-P-dolichol. Interestingly, acceptor peptide also increased the activities in cells not treated with cycloheximide (group 3). This suggests that the free dolichol-P in these cells was supplemented by dolichol-P resulting from discharge of Glc₃Man₉GlcNAc₂-P-P-dolichol.

As shown with CHO-K1 cells not treated with cycloheximide in Figure 9, panel A, the enhancement due to acceptor peptide was substantial for the first 15 minutes, and then followed by a more modest rate of product accumulation. On the other hand, in the presence of non-acceptor peptide GPT product accumulated at a modest rate throughout the entire experiment. Calculation of the difference between the two graphs shows that net effect of the acceptor peptide was limited to the first 15 minutes of the experiment. As determined by FACE analysis, acceptor peptide discharged approximately 60\% of the LLO. Discharge was nearly complete after 5 minutes, and preceded enhancement of GPT activity. This may reflect the time necessary for dephosphorylation of dolichol-P-P and return of dolichol-P to the cytoplasmic leaflet.

After treatment of CHO-K1 cells with SLO and incubation in the absence of peptide (Figure 9, panel B), or in the presence of either acceptor or non-acceptor peptide (not shown), much of the
Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (lane 1) was converted to a species behaving as Man$_9$GlcNAc$_2$-P-P-dolichol (lane 2). This conversion occurred after the SLO treatment step, during the subsequent 30 minute incubation with transport buffer (not shown). In other experiments, no changes were noticed for Man$_5$GlcNAc$_2$-P-P-dolichol from similarly treated Lec15 and Lec35 cells. The appearance of Man$_9$GlcNAc$_2$-P-P-dolichol in permeabilized CHO-K1 cells could be prevented by including UDP-glucose in the transport buffer (lane 3), or by pre-incubation of cells with castanospermine (lane 4). The Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol preserved by castanospermine pretreatment was a competent donor substrate with acceptor peptide (not shown). Taken together, these data indicate that ER glucosidases I and II acted upon the Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol during the incubation with transport buffer, in a manner previously reported (35).
Discussion

These results provide strong support for the 1980 proposal of Hubbard and Robbins (16) for a limited pool of dolichol-P (Figure 1), and show that GPT, MPDS, and GPDS are all controlled by a common mechanism. Further, the limited pool is a primary source of dolichol-P. The SLO system allows the regulation of these key reactions in the dolichol cycle to be studied fastidiously in vitro, apparently by preventing intermingling of the limited primary pool with a secondary pool of dolichol-P.

Discrepancies with prior studies-- Grant and Lennarz (18) found that translation inhibitors did not interfere with the syntheses of GlcNAc-P-P-dolichol, GlcNAc₂-P-P-dolichol, or Man₁GlcNAc₂-P-P-dolichol in intact cells using labeled sugar precursors. Pan and Elbein (20) noted some reduction of GlcNAc-P-P-dolichol synthesis by translation inhibitors, but only with treatment times and concentrations above those necessary to block both translation and LLO synthesis. In part, these prior assessments of GlcNAc-P-P-dolichol synthetic rates in vivo may have been complicated by factors such as the reversibility of the GPT reaction and the ability of MPD to stimulate GPT (10).

Two groups found that dolichol-P added to the culture medium stimulated [³H]Glc₃Man₉GlcNAc₂-P-P-dolichol synthesis in untreated cells, but not in cells treated with translation inhibitors (18,20). The interpretation was that the inhibition of LLO synthesis was not due to a lack of dolichol-P. In both experiments, cells were incubated with [2-³H]mannose. However, it was also found that exogenous dolichol-P did not stimulate [³H]Glc₃Man₉GlcNAc₂-P-P-dolichol synthesis in untreated cells if [³H]glucosamine was used (20). Further, while exogenous dolichol-P stimulated [³H]MPD synthesis in untreated cells incubated with [³H]mannose,
[^3]H\text{GlcNAc}_2\text{P}-\text{dolichol}
synthesis from [^3]H\text{glucosamine}
was not stimulated (20). Such results
suggested the existence of two functional pools of dolichol-P:
one used for LLO initiation by GPT
that is not increased with exogenous dolichol-P, and a second pool used for LLO extension with
MPD that is increased by exogenous dolichol-P. However, in the present study, GPT and MPDS
were found to use the same limited pool of dolichol-P.

It is therefore important to realize that in the study reported here and in that of Hubbard and
Robbins (16), LLO synthesis inhibition was at least 98\%, and effects of inhibitors were observed
within 15 min of addition and lasted for at least 1 hr. In the other two series of studies, inhibition of
LLO synthesis was generally 70-85\%. Further, in one case effects were reported within 5 minutes,
but significant resumption of LLO synthesis occurred after 40 min (18). In the other case, effects
were not apparent until 2 hours after addition of inhibitors, and were then stable for at least an
additional 6 hours (17). The model in Figure 1 may therefore only apply for robust inhibition of
translation, while other factors might account for the results observed with milder inhibition of LLO
synthesis. For example, under the conditions employed here, inhibition of LLO initiation would
prevent the formation of Man\text{2,4GlcNAc}_2\text{P}-\text{dolichol} that might otherwise accumulate with less
complete inhibition of translation as hypothesized (18). The use of suspension cultures (18) may
introduce additional variables because as noted in the Results, suspension of CHO-K1 cells
partially relieved inhibition of LLO synthesis.

\textit{The SLO system is highly preferable for LLO in vitro studies}\textemdash Since microsomal systems
are generally considered to be reliable for studying LLO biosynthetic enzymes, it was surprising to
find that the effects of translation blockers on the activities of GPT, MPDS, and GPDS, as well as
the limitation on activity of overexpressed GPT, were obscured in microsomes. By selective
permeabilization of the plasma membrane with SLO, effects of translation blockers on LLO
synthesis previously reported in vivo were observed in vitro. Interestingly, the possibility was raised before that cell disruption might affect the detection of a regulated state important for coupling of glycosylation with translation (20). In addition, the inactivity of overexpressed GPT in the SLO system compared with microsomes (Figure 5) provided an explanation for its inability to counteract the effects of cycloheximide on LLO initiation in vivo (Figure 6), and for the absence of increased of LLO quantities in Tn-10 cells compared with other Man₅GlcNAc₂-P-P-dolichol accumulating mutants (Figure 6). Cycloheximide-treated Tn-10 cells assayed for GPT activity in the SLO system showed somewhat higher residual activity (approximately 30%) compared with similarly treated CHO-K1 cells (10-20%). Though the reason is unclear, it is possible that a small fraction of the overexpressed GPT is functional, or that this multi-transmembrane span enzyme actually disrupts the ER membrane and emulates the situation with microsomes. Since the overexpressed GPT in Tn-10 cells is catalytically nonfunctional, it is unlikely that it causes accumulation of Man₅₉GlcNAc₂-P-P-dolichol by consuming the majority of the available dolichol-P and drastically reducing synthesis of MPD and GPD. FACE measurements showed that Tn-10 cells have fairly normal quantities of MPD (N.G. and M.A.L., unpublished), and 3E11 cells can produce about half the normal amounts of [³H]-mevalonate labeled MPD and GPD (32). Thus, the actual basis for the LLO defect in Tn-10 and 3E11 cells remains unclear. These results also indicate that overexpression of GPT mediates resistance to TN by buffering, rather than a compensatory increase in catalytic activity, consistent with prior reports of catalytically inactive forms of GPT that were still able to confer resistance to TN (5,36).

A possible role for potential dolichol recognition sequences (PDRS)--How might a limited primary pool of dolichol-P be formed? One scenario is a membrane domain or "raft" containing both dolichol-P and LLO transferases. Such domains might be easily disturbed upon disruption of
cells, allowing the transferases access to dolichol-P not in rafts. A second possibility involves the previously reported potential dolichol recognition sequence, or PDRS. As reviewed (4), one or two copies of PDRSs are found in transmembrane segments of many transferases required for the early stages of LLO synthesis, and were originally suggested to facilitate binding of the dolichol-conjugate acceptor substrates to the enzyme catalytic sites. Mutations affecting this sequence in *S. cerevisiae* MPDS have widely variable effects on activity of MPDS *in vitro* (37,38), from essentially no effect to 99% inhibition depending upon the exact mutation and the assay procedure. Both PDRSs of hamster GPT are required for activity *in vivo* and *in vitro* (microsomes) (36), but the exact functions of these PDRSs were not clear. The discussions in these various reports all suggested that PDRSs might be required for folding, stability, or sorting of MPDS or GPT as opposed to substrate binding.

Given the results reported here, the function of the PDRS might be to allow each dolichol chain to act as an anchor around which LLO transferases might cluster and assemble the oligosaccharide. The limited pool would consist of only those dolichol-P molecules in such complexes, which would dissociate with perturbation. Some transferases might bind directly to the dolichol chain, and others might bind secondarily to the PDRS-containing enzymes. In this scheme the PDRSs would not be required for binding of acceptor substrate at the catalytic site, but instead facilitate interactions between the enzyme and substrate, and could easily reside in a separate domain of the enzyme. This idea is attractive because it provides an explanation for the great variability in the activities of the various PDRS mutants with the different systems used. It also suggests a basis for nature's use of C_{55-95} polyisoprenol carrier lipids (with many potential anchor sites) as opposed to lipids with shorter chains that are theoretically also capable of tight membrane
association. In this hypothesis, overexpressed GPT would not be functional because the endogenous GPT would be sufficient to occupy all available docking sites.

Potential role of control by translation arrest--While these results prompt a re-evaluation of our understanding of the organization of the components of the LLO pathway, do the effects of translation arrest have physiological relevance? There is good evidence for a role (39) for translation arrest resulting from ER stress due to unfolded protein response (UPR) activation of the PKR-like ER kinase "PERK" (40). Stress-induced translation arrest might prevent new synthesis of LLOs, which would not be needed in the absence of nascent proteins. To achieve sufficient translation arrest to inhibit LLO synthesis, potent ER stress would be required (40). In contrast, the stimulatory effects of ER stress on LLO extension reported earlier (11) occur with concentrations of UPR inducers that cause little or no translation arrest (12), that do not prevent protein N-glycosylation, and that do not inhibit LLO synthesis (11). Taken together, while mild ER stress enhances LLO extension, it may be that strong ER stress inhibits LLO initiation.

Summary--The dolichol-P-P-oligosaccharide pathway is linked to protein synthesis by regulation of the step catalyzed by the initiating enzyme, GPT, in a manner that is obscured by microsome preparation. In this regard, the SLO system presents considerable advantages for studying regulation of LLO synthesis.
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Footnote

1 The abbreviations used were: AMAC, 2-aminoacridone; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; ATCC, American Type Culture Collection; CYC, cycloheximide; FACE, fluorophore-assisted carbohydrate electrophoresis; GPD, glucose-P-dolichol; GPDS, GPD synthase; GPT, GlcNAc-1-P transferase; LLO, lipid-linked oligosaccharide; MPD, mannose-P-dolichol; MPDS, MPD synthase; OT, oligosaccharyltransferase; PBS, phosphate-buffered saline; PDRS, potential dolichol recognition sequence; PUR, puromycin; SLO, streptolysin-O; TN, tunicamycin.
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Legends to Figures

Figure 1. Model for the effects of translation inhibition and homogenization on LLO initiation. The key features of the model are described in the text (Results). The attenuated state of LLO initiation resulting from translation inhibition can be studied in vitro if cells are permeabilized with SLO. However, the attenuated state is masked by physical perturbation, as occurs during the preparation of microsomes.

Figure 2. Effects of translation inhibitors on new synthesis and steady-state amounts of lipid-linked oligosaccharides. 8-10 x 10^6 cells were treated for 1 hour with 0.2 mM cycloheximide (CYC) or 0.4 mM puromycin (PUR). Effects on incorporation of [35S]methionine into total protein (panel A) or incorporation of [3H]mannose into LLO (Man5GlcNAc2-P-P-dolichol plus Glc3Man9GlcNAc2-P-P-dolichol determined by HPLC; panel B) are presented as percentages of untreated controls (CON). Results shown are the averages of 2 determinations and are typical of 2 independent experiments. FACE was used to determine the compositions of LLOs in the CMW 10:10:3 (panel C) and CM 2:1 (panel D) fractions. For panel C, an oligosaccharide profiling gel was used. Lane 1, control (CON). Lane 2, cycloheximide treatment (CYC). Lane 3, puromycin treatment (PUR). Standards (STD) in lane 4 were a mixture of Glc0-3Man5-GlcNAc2-ANTS and Man1GlcNAc2-ANTS (21). The standard mixture also included a partial jack bean α-mannosidase digest of Man5GlcNAc2-ANTS, to identify the portion of the gel where Man2-GlcNAc2-ANTS would be expected to run. For panel D, a monosaccharide composition gel was used. Lane 3, control (CON). Lane 4, cycloheximide (CYC). Standards were either (lane 1) chitobiosyl-AMAC (GlcNAc2-AMAC, double arrowhead), or (lane 2) chitobiosyl-AMAC mixed with GlcNAc-AMAC.
Based upon additional FACE analyses, the three most prominent species in the CON and CYC lanes were tentatively assigned as a co-migrating mixture of D-glucosyl-AMAC and D-galactosyl-AMAC (Glc/Gal), a comigrating mixture of D-mannosyl-AMAC and L-fucosyl-AMAC (Man/Fuc), and an unknown (?)

Figure 3: Effects of cycloheximide treatments on GlcNAc-1-P transferase assayed in either microsomes or SLO-permeabilized cells. Panel A: CHO-K1 cells were treated in the absence (lanes 1,2,5,6) or presence (3,4,7,8) of 0.2 mM cycloheximide for 1 hour. 8-10 x 10^6 cells were either homogenized for microsome preparation, or permeabilized with SLO. The same cell-equivalents were incubated at 37 deg. for 15 min with 2 ml of transport buffer containing 0.2 µCi/ml UDP-[^3H]GlcNAc, in the absence (lanes 1,3,5,7; open bars) or presence (lanes 2,4,6,8; filled bars) of tunicamycin (10 ng/ml for microsomes, 10 µg/ml for SLO-treated cells). GPT activity was determined as described under Experimental Procedures, and expressed as c.p.m. of[^3H]GlcNAc incorporated into lipid. Results shown are the averages of 3 determinations and are typical of 4 independent experiments. Panel B: GPT activity in Lec15.2 cells treated with cycloheximide (lanes 2 and 4) was analyzed as described for panel A, and expressed as percentage of untreated controls (lanes 1 and 3), which gave average values of 400 cpm/8-10 x 10^6 cell equivalents.

Figure 4: Effect of time of cycloheximide treatment on LLO synthesis and GPT activity in SLO-permeabilized cells. (Panel A) CHO-K1 cells were treated with cycloheximide for the times indicated, and permeabilized with SLO. One set of cells was assayed for synthesis of Man_9GlcNAc_2-P-P-dolichol in the presence of 1 µM UDP-GlcNAc and 0.1 µCi/ml GDP-
[\textsuperscript{3}H]mannose, followed by a 5 min chase with 10 \textmu M GDP-mannose and analysis of Man\textsubscript{9}GlcNAc\textsubscript{2}-P-P-dolichol by HPLC (LLO, filled bars). A second set was assayed for GPT activity (GPT; open bars). Results shown are the averages of 2 determinations. (Panel B) HPLC profiles showing LLOs detected for panel A. A: 0 minutes cycloheximide treatment; B: 15 minutes; C: 30 minutes; D: 60 minutes. In all cases Man\textsubscript{9}GlcNAc\textsubscript{2}-P-P-dolichol was produced, indicating the availability of MPD.

**Figure 5:** Activity of exogenously expressed GPT in Tn-10 cells is detected in microsomes but not after permeabilization with SLO. (Panel A) GPT activity (net cpm/assay) from 8-10 x 10\textsuperscript{6} control (filled bars) or cycloheximide-treated (open bars) Tn-10 cells was assayed after preparation of microsomes (left bars) or after permeabilization with SLO (right bars). For open bars, the percent of untreated control is given in parentheses. In separate controls (data not shown), the GPT activity in microsomes from untreated Tn-10 cells was approximately 10-fold greater than in untreated CHO-K1 microsomes as reported earlier (22). The GPT activities of the two types of SLO-permeabilized cells compared directly were similar (CHO-K1, 374 cpm; Tn-10, 431 cpm), as expected by comparison of Figures 3 and 5. Results shown are the averages of 2 determinations and are typical of 4 independent experiments. (Panel B) 8-10 x 10\textsuperscript{6} Tn-10 cells were treated with cycloheximide for the times indicated, permeabilized with SLO, and assayed for either LLO synthesis (closed bars) or GPT (open bars) as described for Figure 4, panel A. Results shown are the averages of 2 determinations and are typical of 3 independent experiments.

**Figure 6.** LLO synthesis in Tn-10 cells. 4 x 10\textsuperscript{7} cells were incubated in the absence or presence of 0.2 mM cycloheximide for 60 min, and LLOs were characterized as described under *Experimental*.
Procedures. Panel A: FACE analysis. Lanes 1 and 6, CHO-K1 cells. Lane 2, Tn-10 cells. Lane 3, Tn-10 cells treated with cycloheximide. Lane 4, mouse liver LLOs as standards. Lane 5, Lec35.1 cells. Panel B: CHO-K1 (squares) and Tn-10 (circles) cells were treated with cycloheximide for the times indicated, incubated with $[^3]$H]mannose for 20 minutes, and total $[^3]$H]LLO in the CMW 10:10:3 extract was measured.

Figure 7. GPT and MPDS compete for the same limited pool of dolichol-P. 100 mM dishes containing $10^7$ CHO-K1 (upper graph) or Lec15.2 (lower graph) cells were treated with SLO. They were then subjected to three sequential incubations of 10, 20, and 10 minutes each at 37 degrees as indicated (groups 1-3), with PBS washes before the 2nd and 3rd incubations. Incubations contained either transport buffer alone, buffer plus 1 mM unlabeled GDP-mannose, or buffer plus 0.4 $\mu$Ci/ml UDP-$[^3]$H]GlcNAc. The cells were harvested in methanol and dried under nitrogen gas. Graphs show $[^3]$H]GlcNAc-P-P-dolichol (light gray bars) and $[^3]$H]-LLO (dark gray bars), prepared as described under Experimental Procedures, the $[^3]$H]-protein residue (black bars) remaining after LLO extraction, and the arithmetic sum of $[^3]$H in all three glycoconjugate fractions (white bars). In all cases, the data are reported at c.p.m. determined by liquid scintillation spectroscopy and are the averages of duplicates.

Figure 8. Acceptor peptide for oligosaccharyltransferase added to SLO-permeabilized cells after cycloheximide treatment restores the in vitro activities of GPT, MPDS, and GPDS. 10 cm dishes containing $10^7$ CHO-K1 cells were treated in the absence (groups 1-3) or presence (groups 4-6) of 0.2 mM cycloheximide for 1 hr. Cells were permeabilized with SLO, and then incubated at 37 degrees for 30 minutes in the absence (groups 1 and 4) or presence of 50 $\mu$M control (non-acceptor,
groups 2 and 5) or acceptor (groups 3 and 6) tripeptide and the appropriate tritium-labeled nucleotide-sugar to assay GPT (upper panel), MPDS (center panel), or GPDS (lower panel). Data are the averages (+/- S.E.) from two independent experiments, and are plotted as the percentage of activity for group 1.

Figure 9. Enhancement of GPT activity in SLO-permeabilized cells by acceptor peptide corresponds to discharge of Glc$_{0.3}$Man$_9$GlcNAc$_2$-P-P-dolichol. Panel A: Using CHO-K1 cells that were not treated with cycloheximide but permeabilized with SLO, incubations with either 50 µM non-acceptor peptide (closed circles) or acceptor peptide (closed squares) were performed as described for groups 2 and 3 of Figure 8, and GPT activity (c.p.m.) was plotted. The closed triangles show the arithmetic difference between these two lines. The percent of total Glc$_{0.3}$Man$_9$GlcNAc$_2$-P-P-dolichol discharged by acceptor peptide was measured by FACE (open triangles). Essentially no discharge was detected with non-acceptor peptide (not shown).

Panel B: LLOs were assessed by FACE in CHO-K1 cells not treated with cycloheximide. Lane 1: intact cells. Lane 2: cells permeabilized with SLO and then incubated in transport buffer for 30 minutes. Lane 3: as for lane 2, but with 0.2 mM UDP-glucose included during the incubation. Lane 4: as for lane 2, with addition of 200 µg/ml castanospermine to both the transport buffer and to the growth medium for the final 2 hours prior to SLO permeabilization. The positions of Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol (G$_3$M$_9$) and Man$_9$GlcNAc$_2$-P-P-dolichol (M$_9$) are indicated.
Table I. Cycloheximide treatments of intact cells reduce the activities of three dolichol-P dependent transferases when assayed with SLO in vitro systems, but not microsomal systems.

10^7 CHO-K1 cells were treated with 0.2 mM cycloheximide for 1 hr, either permeabilized with SLO or homogenized for preparation of microsomes, and assayed for activity of each of three dolichol-P dependent transferases using endogenous acceptor as described under Experimental Procedures. Activities are reported as percentages of those obtained without cycloheximide treatment. These values in c.p.m. for the SLO and microsomal systems were, respectively 485 and 459 (GPT), 952 and 975 (MPDS), and 508 and 1130 (GPDS). GPT activity (SLO system) from untreated cells was reduced by 93 % with TN. MPDS activity (SLO system) in untreated Lec15.2 cells was 10 % of that in untreated CHO-K1 cells. No comparable control was available for GPDS. However, for MPDS and GPDS, activities were determined after organic extraction and enrichment of phosphomonoester products by DEAE-cellulose chromatography (2 mM NaOAc eluate) to eliminate neutral mannosyl and glucosyl lipids.

| Enzyme | In vitro enzyme activity from cycloheximide-treated cells (percent of activity in untreated cells) |
|--------|--------------------------------------------------------------------------------------------------|
|        | SLO system                                                                                       |
| GPT    | 15 ± 4 (n=20)                                                                                     |
| MPDS   | 18 ± 2 (n=4)                                                                                      |
| GPDS   | 30 ± 6 (n=4)                                                                                      |
|        | Microsomal system                                                                                |
| GPT    | 127 ± 19 (n=5)                                                                                     |
| MPDS   | 110 ± 13 (n=2)                                                                                     |
| GPDS   | 123 ± 21 (n=2)                                                                                     |
Table II. Oligosaccharyltransferase acceptor peptide added to intact cells counteracts the cycloheximide-induced reductions of LLO synthesis in vivo and GPT in the SLO in vitro system.

In 6-well plates, duplicate sets of wells containing 10^6 CHO-K1 cells were treated with 0 or 0.2 mM cycloheximide for 1 hr, in the absence or presence of peptide. For one set, cells were incubated with [2-^3H]mannose for 20 min and total[^3H]-LLO was measured. The other set was permeabilized with SLO and assayed for GPT activity as described under Experimental Procedures. Activities are reported as percentages of those for cells not treated with cycloheximide or peptide (top line of table).

| Cycloheximide treatment mM | Gln-Tyr-Thr (Non-acceptor for O.T.) µM | Asn-Tyr-Thr (Acceptor for O.T.) µM | LLO synthesis in intact cells percent of control | GPT Activity in SLO system percent of control |
|---------------------------|---------------------------------------|-------------------------------------|-----------------------------------------------|---------------------------------------------|
| 0                         | 0                                     | 0                                  | 100                                           | 100                                         |
| 0.2                       | 0                                     | 0                                  | 2.7                                           | 18                                          |
| 0.2                       | 50                                    | 0                                  | 2.0                                           | 21                                          |
| 0.2                       | 500                                   | 0                                  | 2.7                                           | 33                                          |
| 0.2                       | 0                                     | 50                                 | 3.4                                           | 257                                         |
| 0.2                       | 0                                     | 500                                | 11.3                                          | 246                                         |
Figure 1
Figure 2

A. *In Vivo* PROTEIN SYNTHESIS

B. *In Vivo* LLO SYNTHESIS

C. LLO (CMW 10:10:3)

D. LLO (CM 2:1)
Figure 3

A. CHO-K1

Microsomes:  
SLO:

GPT Activity (cpm)

Cycloheximide:  -    -    +    +           -    -    +    +
Tunicamycin: -    +    -    +               -    +   -     +

B. Lec15

Microsomes:  
SLO:

Cycloheximide:  -    +                -    +

GPT Activity (percent of control)
Figure 4

A.

![Bar graph showing synthesis in vitro (percent of control) for Cycloheximide Treatments](image)

B.

![Chromatogram showing [3H]Detector Response for M₉Gn₂](image)
Figure 5

Tn-10 cells:

A.

GPT Activity (c.p.m.)

Control

Cycloheximide

(108%)

Microsome

SLO

B.

Synthesis In vitro (percent of control)

0 min                  15 min                 30 min                 60 min

LLO

GPT

Cycloheximide Treatments
Figure 6

A.

| CHO-K1 | Tn-10 | Tn-10 + cyclohex. | LLO standards | Lec35 | CHO-K1 |
|--------|-------|-------------------|---------------|-------|--------|
|        |       |                   |               |       | Glc3Man9GlcNAc2 |
|        |       |                   |               |       | Man9GlcNAc2 |
|        |       |                   |               |       | Man5GlcNAc2 |

1 2 3 4 5 6

B.

[^3]H] LLO Synthesis In Vivo (percent of control) vs. Time of Cycloheximide Treatment (minutes)

- CHO-K1
- Tn-10
Figure 7

| Group  | 1st incubation | 2nd incubation | 3rd incubation |
|--------|----------------|----------------|----------------|
| CHO-K1 | Buffer         | UDP-[^3]H]GlcNAc | Buffer         |
|        | [^3]H]GlcNAc-P-P-dolichol | [^3]H]GlcNAc-P-P-dolichol | [^3]H]-LLO |
|        | [^3]H]-LLO | [^3]H]-LLO | [^3]H]-LLO |
| Lec15.2| Buffer         | UDP-[^3]H]GlcNAc | GDP-man        |
|        | [^3]H]-LLO | [^3]H]-LLO | [^3]H]-LLO |
|        | [^3]H]-LLO | [^3]H]-LLO | [^3]H]-LLO |
Figure 8

Enzyme Activity (relative percent)

GPT

MPDS

GPDS

|   | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| cycloheximide |   |   |   |   |   |   |
| control peptide | + | + | + |   |   |   |
| acceptor peptide | + |   |   | + | + | + |
Figure 9

A.

GPT Activity (c.p.m.)

Time (minutes)

B.

SLO \textit{in vitro} system

|   | Intact | ----- | UDP-glc | CSN |
|---|--------|-------|---------|------|
| 1 |        |       |         |      |
| 2 |        |       |         |      |
| 3 |        |       |         |      |
| 4 |        |       |         |      |

$G_{0,3-MGn2-P-P-Dolichol}$

Discharged (percent)

\[ \Delta \]
Coupling of the dolichol-P-P-oligosaccharide pathway to translation by perturbation-sensitive regulation of the initiating enzyme, GlcNAc-1-P transferase
Ningguo Gao and Mark A. Lehrman

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