Large-scale isolation of dolichol-linked oligosaccharides with homogeneous oligosaccharide structures: determination of steady-state dolichol-linked oligosaccharide compositions

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The dolichol-linked oligosaccharide donor (Glc₃Man₉GlcNAc₂-PP-Dol) for N-linked glycosylation of proteins is assembled in a series of reactions that initiate on the cytoplasmic face of the rough endoplasmic reticulum and terminate within the lumen. The biochemical analysis of the oligosaccharyltransferase and the glycosyltransferases that mediate assembly of dolichol-linked oligosaccharides (OS-PP-Dol) has been hindered by the lack of structurally homogeneous substrate preparations. We have developed an improved method for the preparative-scale isolation of dolichol-linked oligosaccharides from vertebrate tissues and yeast cells. Preparations that were highly enriched in either Glc₃Man₉GlcNAc₂-PP-Dol or Man₉GlcNAc₂-PP-Dol were obtained from porcine pancreas and a Man₉GlcNAc₂-PP-Dol preparation was obtained from an αalg3 yeast culture. Chromatography of the OS-PP-Dol preparations on an aminopropyl silica column was used to obtain dolichol-linked oligosaccharides with defined structures. A single chromatography step could achieve near-baseline resolution of dolichol-linked oligosaccharides that differed by one sugar residue. A sensitive oligosaccharyltransferase endpoint assay was used to determine the concentration and composition of the OS-PP-Dol preparations. Typical yields of Glc₃Man₉GlcNAc₂-PP-Dol, Man₉GlcNAc₂-PP-Dol, and Man₉GlcNAc₂-PP-Dol ranged between 5 and 15 nmol per chromatographic run. The homogeneity of these preparations ranged between 85 and 98% with respect to oligosaccharide composition. Purification of dolichol-linked oligosaccharides from cultures of αlG mutants yeast strains provides a general method to obtain authentic OS-PP-Dol assembly intermediates of high purity. The analytical methods described here can be used to accurately evaluate the steady-state dolichol-linked oligosaccharide compositions of wild-type and mutant cell lines.

Key words: N-linked glycosylation/lipid-linked oligosaccharides/glycosyltransferases/rough endoplasmic reticulum/dolichol/αlG mutants

Introduction

The dolichol-linked oligosaccharide Glc₃Man₉GlcNAc₂-PP-Dol is the oligosaccharide donor for N-linked glycosylation of proteins in all eukaryotic organisms except trypanosomatid protozoa (Parodi, 1993), which utilize nonglucosylated oligosaccharide donors for glycoprotein biosynthesis. The assembly pathway for Glc₃Man₉GlcNAc₂-PP-Dol has been deduced from the analysis of intermediates that accumulate in vitro in membranes from wild type cells (Chapman et al., 1979; Liu et al., 1979) and by the analysis of the yeast αlG mutants (Huffaker and Robbins, 1983) that lack glycosyltransferase activities (for a review see Burda and Aebi, 1999). Glc₃Man₉GlcNAc₂-PP-Dol assembly is initiated on the cytoplasmic face of the rough endoplasmic reticulum (RER) where UDP-N-acetylgalactosamine and GDP-mannose serve as sugar donors for the glycosyltransferases that mediate the stepwise assembly of Man₉GlcNAc₂-PP-Dol (Abeijon and Hirschberg, 1990). The final four mannose residues and the three glucose residues are transferred by glycosyltransferases that face the RER lumen and use Man-P-Dol and Glc-P-Dol as the sugar donors. Although the majority of the yeast genes that encode glycosyltransferases involved in Glc₃Man₉GlcNAc₂-PP-Dol assembly have been identified (Burda and Aebi, 1999), relatively few of these glycosyltransferases have been biochemically characterized in any organism (Sharma et al., 1990; Datta and Lehman, 1993; Mudgapalli et al., 1994). The relatively few biochemical studies of the dolichol assembly pathway enzymes are explained at least in part by the lack of pure dolichol-linked oligosaccharides that can be used as substrates for the glycosyltransferases. Although the oligosaccharyltransferase (OST) preferentially utilizes the fully assembled donor, a broad spectrum of assembly intermediates (GlcNAc₂-PP-Dol to Glc₃Man₉GlcNAc₂-PP-Dol) can also be utilized in vitro and in vivo (Sharma et al., 1981; Jackson et al., 1989). Biochemical quantities of homogeneous dolichol-linked oligosaccharides are required to investigate the kinetic and mechanistic features of the oligosaccharyltransferase.

Methods to prepare dolichol-linked oligosaccharides from tissues or cells using sequential extractions with organic solvent mixtures were first described nearly 30 years ago (Parodi et al., 1972). Modified versions of the initial extraction procedure have been extensively used to prepare radiolabeled dolichol-linked oligosaccharides from tissue slices, tissue culture cells, and fungal cultures. The predominant dolichol-linked oligosaccharide detected in extracts prepared from δH mannose-labeled wild type cells is Glc₃Man₉GlcNAc₂-PP-Dol (D’Souza et al., 1992; Aebl et al., 1996). In contrast, large-scale OS-PP-Dol isolates are much more heterogeneous and contain a preponderance of compounds that lack glucose
residues (Badet and Jeanloz, 1988; Gibbs and Coward, 1999). For example, an OS-PP-Dol preparation isolated from calf pancreas consisted of compounds ranging between Man₇GlcNAc₂-PP-Dol and Glc₃Man₅GlcNAc₂-PP-Dol, with the most abundant species being Man₅GlcNAc₂-PP-Dol (Badet and Jeanloz, 1988). Complex mixtures of radiolabeled dolichol-linked oligosaccharides have been resolved by high-pressure liquid chromatography (HPLC) on a silica gel column (Wells et al., 1981). A partial resolution of radiolabeled Man₅GlcNAc₂-PP-Dol from Glc₃Man₅GlcNAc₂-PP-Dol has been achieved by anion exchange chromatography (Spiro et al., 1979b). Digestion of 3H mannose-labeled Glc₃Man₉GlcNAc₂-glycosyltransferases. Specific dolichol-linked oligosaccharides pathway, hence they may not be optimal substrates for the do not correspond to authentic intermediates in the assembly Man₅GlcNAc₂-PP-Dol, the majority of the isolated compounds do not correspond to authentic intermediates in the assembly pathway, hence they may not be optimal substrates for the glycospinosyltransferases. Specific dolichol-linked oligosaccharides have been prepared by organic synthesis (GlcNAc₂-PP-Dol; Lee and Coward, 1992) or by large-scale in vitro biosynthesis of the dolichol-linked oligosaccharide followed by multiple rounds of anion exchange chromatography (GlcNAc₂-PP-Dol and Man₅GlcNAc₂-PP-Dol; Kaushal and Elbein, 1986; Sharma et al., 1990).

We have developed a general method for the large-scale isolation of purified dolichol-linked oligosaccharides from mammalian tissues or yeast cells. The isolation procedure was modified to reduce hydrolysis of the oligosaccharides during early steps of the procedure. We have developed a rapid HPLC procedure to resolve dolichol-linked oligosaccharides on the basis of oligosaccharide size. Purification of Man₅GlcNAc₂-PP-Dol from an Δαlg₃ yeast culture demonstrates the utility of this method for the preparation of pure dolichol-linked oligosaccharide assembly intermediates. Detection of the novel compound Glc₃Man₅GlcNAc₂-PP-Dol in an OS-PP-Dol preparation isolated from Δαlg₃ yeast cells demonstrates that the dolichol-linked oligosaccharide purification method and the OST endpoint assay described here provide powerful analytical procedures to analyze dolichol-linked oligosaccharide biosynthesis in wild-type and mutant cells.

Results

Composition of dolichol-linked oligosaccharide preparations

The objective of this study was to develop a procedure to isolate biochemical quantities of homogeneous dolichol-linked oligosaccharides from vertebrate tissues or yeast cells. As a first step toward this goal we needed to devise a sensitive method to determine the concentration and oligosaccharide composition of crude OS-PP-Dol preparations. The yield and oligosaccharide composition of the glycopeptide products from an oligosaccharyltransferase (OST) assay that was allowed to go to completion should reflect the concentration and composition of the donor substrate. The yeast OST was used for this assay due to a reduced discrimination, relative to the canine enzyme, between fully assembled and incompletely assembled donor substrates (Kelleher, Karaoglu, and Gilmore, unpublished data). Purified yeast oligosaccharyltransferase (Kelleher and Gilmore, 1994), which lacks glucosidase or mannosidase activities, was incubated with bovine dolichol-linked oligosaccharides and the iodinated tripeptide Nα-Ac-N-[125I]Y-T-NH₂ under conditions that support glycopeptide synthesis. Quantitation of the glycopeptide products that were collected on ConA Sepharose beads at time points ranging between 0 and 48 h showed that glycopeptide formation reached a plateau between 24 and 48 h (Figure 1A, circles). When more donor substrate was added to an assay after 46 h, the yield of glycopeptide was much greater at the 47-h time point (Figure 1A, filled square), indicating that the OST remained active and that the acceptor peptide was present in excess relative to the dolichol-linked oligosaccharide. In contrast, the addition of a second aliquot of OST at the 24-h time point did not significantly alter the yield of glycopeptide.
products that were recovered at the 47-h time point (Figure 1A, triangle). Preincubation of an endpoint assay for 1–8 h prior to adding the OST did not significantly reduce the final yield of glycopeptide product, indicating that the donor substrate was stable in our assay (not shown). The concentration of functional dolichol-linked oligosaccharide in an OS-PP-Dol preparation was calculated from the yield of glycopeptide products obtained in the OST endpoint assay and the known specific activity of the iodinated tripeptide acceptor (15,000 cpm/pmold). Monosaccharide analysis of acid-hydrolyzed OS-PP-Dol samples confirmed that the OST endpoint assay provides a reliable quantification of the OS-PP-Dol concentration (see Monosaccharide analysis of purified OS-PP-Dol).

The glycopeptide products from OST endpoint assays were eluted from the ConA Sepharose beads with acidified acetonitrile and subsequently resolved on the basis of oligosaccharide size by HPLC (Meliss and Baenziger, 1983). The elution position of neutral glycopeptides on the aminopropyl silica column is proportional to the number of saccharide residues. In preliminary studies when a bovine OS-PP-Dol preparation was used as the donor substrate, gamma counting of the eluted fractions revealed a heterogeneous mixture of glycopeptide products (Figure 1B, see inset). The latest eluting peak was identified as Glc3Man9GlcNAc2-NYT (G3) based on the more rapid kinetics of formation relative to all other products (data not shown). The earlier eluting glycopeptides carry oligosaccharides ranging in size between Man5GlcNAc2 (M3) and Glc3Man9GlcNAc2-NYT. Glc3Man9GlcNAc2-NYT accounted for less than 10% of the total products. The oligosaccharide composition of the glycopeptide products did not vary between the 24- and 48-h endpoint assays, nor did the inclusion of deoxyxojirimycin, a glucosidase inhibitor, increase the percentage of Glc3Man9GlcNAc2-NYT (data not shown). Notably, the composition of this bovine OS-PP-Dol preparation was similar to that reported previously (Badet and Jeanloz, 1988; Gibbs and Coward, 1999) with respect to the low abundance of Glc3Man9GlcNAc2-PP-Dol relative to Man5GlcNAc2-PP-Dol. When dolichol-linked oligosaccharide compositions are determined by 3H mannose pulse-labeling of tissue slices, cultured mammalian cells, or yeast cells, the preparation was used as the donor substrate, gamma counting of the eluted products (data not shown). The earlier eluting glycopeptides carry oligosaccharides ranging in size between Man5GlcNAc2 (M3) and Glc3Man9GlcNAc2-NYT. Glc3Man9GlcNAc2-NYT accounted for less than 10% of the total products. The oligosaccharide composition of the glycopeptide products did not vary between the 24- and 48-h endpoint assays, nor did the inclusion of deoxyxojirimycin, a glucosidase inhibitor, increase the percentage of Glc3Man9GlcNAc2-NYT (data not shown). Notably, the composition of this bovine OS-PP-Dol preparation was similar to that reported previously (Badet and Jeanloz, 1988; Gibbs and Coward, 1999) with respect to the low abundance of Glc3Man9GlcNAc2-PP-Dol relative to Man5GlcNAc2-PP-Dol. When dolichol-linked oligosaccharide compositions are determined by 3H mannose pulse-labeling of tissue slices, cultured mammalian cells, or yeast cells, the predominant radiolabeled lipid-linked oligosaccharide is Glc3Man9GlcNAc2-PP-Dol (Spiro and Spiro, 1991; Aebi et al., 1996; Quellhorst et al., 1999). Given this disparity in oligosaccharide composition between large- and small-scale preparations, we hypothesized that the major compounds in this bovine OS-PP-Dol preparation were degradation products rather than assembly intermediates. The glycopeptide products obtained with this bovine OS-PP-Dol preparation were used as Hex12GlcNAc2-NYT mobility standards for subsequent HPLC experiments (HexGlcNAc2-NYT is Man9GlcNAc2-NYT).

The solvent extraction procedure for the large-scale isolation of dolichol-linked oligosaccharides (Kelleher et al., 1992) was modified to increase the yield of the fully assembled donor oligosaccharides and to reduce contamination of the preparation with amphipathic compounds that are derived from other intracellular organelles. To achieve these objectives, a crude microsomal membrane fraction was isolated from porcine pancreas under isotonic conditions to minimize exposure of the OS-PP-Dol compounds to cytoplasmic and lysosomal glycosidases. As described in detail in the methods section, ice-cold solvents were used for the initial solvent extractions. When the composition of the porcine pancreas OS-PP-Dol preparation was determined by HPLC as described above, more than 70% of the glycopeptide products were derived by transfer of the fully assembled dolichol-linked oligosaccharide (Figure 1C). The OST endpoint assay indicated a yield of 0.93 nMol of OS-PP-Dol per g of tissue, which is comparable to that reported previously (Badet and Jeanloz, 1988). Notably, the modified extraction procedure did not reduce the total yield of dolichol-linked oligosaccharides, arguing strongly against the selective loss of dolichol-oligosaccharide assembly intermediates or degradation products using this procedure.

The RER luminal enzymes glucosidase I and II have been shown to trim the glucose residues from Glc3Man9GlcNAc2-PP-Dol in vivo when protein synthesis is blocked by cellular ATP depletion (Spiro et al., 1983; Spiro and Spiro, 1991). A portion of the crude porcine microsome preparation was incubated for 1 h at 37°C in the absence of ATP, sugar nucleotides, and glucosidase inhibitors. When the composition of the dolichol-linked oligosaccharides isolated from the preincubated microsomes was determined using the OST endpoint assay followed by HPLC analysis (Figure 1D), it was apparent that the majority of the Glc3Man9GlcNAc2-PP-Dol had been processed to Man9GlcNAc2-PP-Dol by removal of the glucose residues.

Resolution of dolichol-linked oligosaccharides by ion exchange chromatography

Radiolabeled quantities of Man9GlcNAc2-PP-Dol can be resolved from Glc3Man9GlcNAc2-PP-Dol on a DEAEC-cellulose column equilibrated in CHCl3:CH3OH:H2O (10:10:3) using an increasing linear gradient of CHCl3:CH3OH:0.1 M NH4OAc (10:10:3) (Spiro et al., 1979b). To improve the resolution between different dolichol-linked oligosaccharides, the crude preparations characterized in Figures 1C and 1D were applied to a preparative (24 ml) aminopropyl silica HPLC column equilibrated in CHCl3:CH3OH:H2O (10:10:3). Dolichol-linked oligosaccharides bind to the HPLC column, and most contaminants were recovered in the flow through and wash fractions (see below). The OS-PP-Dol compounds were eluted with a 320 ml linear gradient between solvent A (CHCl3:CH3OH:H2O, 10:10:3) and 40% solvent B (CHCl3:CH3OH:2 M NH4OAc, 10:10:3). A sensitive and rapid ECL-based ConA binding assay was used to detect compounds with terminal glucose or mannose residues in the column eluate fractions (Figure 2A, 2B). The fractions were also assayed using the OST endpoint assay to determine more precisely the distribution and concentration of donor substrates in the eluate fractions (Figures 2C and 2D). When the Glc3Man9GlcNAc2-PP-Dol preparation was applied to the HPLC column, the major peak of ConA binding material eluted between fractions 66 and 74 (Figure 2A). These fractions also contained the majority of the donor substrate that could be detected with the OST endpoint assay (Figure 2C). The ConA binding assay is a semiquantitative procedure used to survey the elution profile during methods development. When equal-sized aliquots of the column fraction are assayed for ConA binding activity, the ECL signal for the most concentrated samples may not fall within the linear range. We also detect a greater ConA binding signal for Glc3Man9GlcNAc2-PP-Dol than for Man9GlcNAc2-PP-Dol when equal amounts of the OS-PP-Dol are affixed to the tube, suggesting that more than two ConA molecules can bind to branched high-mannose oligosaccharides, as suggested by
Consequently, the ConA binding activity for the less abundant triantennary dolichol-linked oligosaccharides (Man₈GlcNAc₂-PP-Dol to Glc₃Man₉GlcNAc₂-PP-Dol) appears to be overrepresented relative to Man₅GlcNAc₂-PP-Dol and Glc₃Man₉GlcNAc₂-PP-Dol in Figure 2A. As expected, multiple peaks of ConA binding activity eluted between fractions 45 and 75 when the more complex Man₉GlcNAc₂-PP-Dol-enriched sample was applied to the HPLC column (Figure 2B, 2D). The majority of the OS-PP-Dol compounds in the Man₉GlcNAc₂-PP-Dol-enriched sample ranged in size between Man₈GlcNAc₂-PP-Dol and Glc₃Man₉GlcNAc₂-PP-Dol (Figure 3, HPLC load). To determine whether individual dolichol-linked oligosaccharides had been resolved by the preparative HPLC column, the glycopeptide products from the OST endpoint assays (Figure 2D) were eluted and resolved by HPLC (Figure 3A). The major peak from the preparative HPLC column was found to consist primarily of Man₉GlcNAc₂-PP-Dol with traces of Man₈GlcNAc₂-PP-Dol (Figure 3A, F-56 and F-58). Fractions 60 and 62 contained mixtures of Man₈GlcNAc₂-PP-Dol, Glc₃Man₉GlcNAc₂-PP-Dol, and Glc₃Man₉GlcNAc₂-PP-Dol. Fractions 64 and 66 were highly enriched in Glc₃Man₉GlcNAc₂-PP-Dol, though traces of smaller compounds were present. The final peak on the preparative HPLC column (Figure 2D) was highly enriched in Glc₃Man₉GlcNAc₂-PP-Dol (Figure 3, F-69), and the early eluting peak (Figure 2B, F46–F48) is Man₅GlcNAc₂-PP-Dol (not shown).

Peak fractions from preparative HPLC columns were pooled to obtain Glc₃Man₉GlcNAc₂-PP-Dol and Man₉GlcNAc₂-PP-Dol preparations that would be suitable for enzyme kinetics studies of the oligosaccharyltransferase. The oligosaccharide composition and OS-PP-Dol concentration of these pooled fractions was determined with the OST endpoint assay (Figure 3B). Glc₃Man₉GlcNAc₂-PP-Dol preparations typically contained between 90% and 95% Glc₃Man₉GlcNAc₂-PP-Dol, with the remainder being a mixture of Glc₃Man₉GlcNAc₂-PP-Dol and Glc₃Man₉GlcNAc₂-PP-Dol. The Man₉GlcNAc₂-PP-Dol preparation typically contained between 85% and 90% Man₉GlcNAc₂-PP-Dol. In this case, the contaminants were Glc₃Man₉GlcNAc₂-PP-Dol and Man₉GlcNAc₂-PP-Dol. Because the recovery of dolichol-linked oligosaccharides on the preparative HPLC column was excellent (> 95%), the yield per chromatographic run largely depended on the quantity (5–15 nmol) and complexity of the OS-PP-Dol sample applied to the column.
Isolation of dolichol-linked oligosaccharides from Δalg3 yeast cultures

The asparagine-linked glycosylation (alg) mutants of Saccharomyces cerevisiae accumulate discrete OS-PP-Dol assembly intermediates due to mutations in genes encoding the glycosyltransferases that mediate the assembly pathway (for a review see Burda and Aebi, 1999). An OS-PP-Dol preparation isolated from an alg mutant culture should be an excellent source for the facile isolation of a homogeneous assembly intermediate by preparative HPLC. To determine whether a large-scale OS-PP-Dol isolation from an alg mutant culture was feasible, we chose the well-characterized alg3 mutant (Aebi et al., 1996). The alg3 mutant accumulates Man\textsubscript{9}GlcNAc\textsubscript{2}-PP-Dol as the major dolichol-linked oligosaccharide (Huffaker and Robbins, 1983) due to a deficiency in Dol-P-Man:Man\textsubscript{9}GlcNAc\textsubscript{2}-PP-Dol α1,3 mannosyltransferase activity (Verostek et al., 1991). A subsequent structural analysis of N-linked oligosaccharides isolated from an alg3 mutant revealed that a substantial proportion of the protein-linked oligosaccharide is Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}, indicating that the alg3 strain also synthesizes the glucosylated dolichol-linked oligosaccharide Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-PP-Dol (Verostek et al., 1993). Here, we used a Δalg3 mutant created by gene disruption (Aebi et al., 1996) to eliminate traces of the fully assembled dolichol-linked oligosaccharide that can arise using the original alg3 isolate (Verostek et al., 1991).

Dolichol-linked oligosaccharides were isolated from a 10.5-L culture of Δalg3 yeast that was grown to a density of 2.2 (A\textsubscript{600}). An OST endpoint analysis indicated a yield of 42 nMol of OS-PP-Dol from 72 g (wet weight) of Δalg3 yeast cells. The glycopeptide products from the OST endpoint assays were analyzed by HPLC to determine which donor substrates were present (Figure 4A). The three major glycopeptide peaks comigrated with the Hex\textsubscript{7}GlcNAc\textsubscript{2}-NYT, Hex\textsubscript{6}GlcNAc\textsubscript{2}-NYT, and Hex\textsubscript{5}GlcNAc\textsubscript{2}-NYT standards. These three compounds respectively account for 71%, 15%, and 6% of the glycopeptide products. Minor peaks with 4, 6, 7, and 9 hexose units were also detected. Thus, without further purification by HPLC, an OS-PP-Dol preparation isolated from Δalg3 cells contains a number of different assembly intermediates. The elution position and relative abundance of the two major glycopeptide products (Hex\textsubscript{7}GlcNAc\textsubscript{2}-NYT and Hex\textsubscript{5}GlcNAc\textsubscript{2}-NYT) is consistent with their identification as Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT and Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT. The percentage of each oligosaccharide donor that was utilized during a 30-min OST assay was determined by HPLC analysis (Figure 4B). Although roughly 20% of the dolichol-linked oligosaccharides had been utilized as substrates by the 30-min time point, the initial transfer rate for the Hex\textsubscript{7}GlcNAc\textsubscript{2}-PP-Dol and Hex\textsubscript{5}GlcNAc\textsubscript{2}-PP-Dol donors was more rapid. As triglucosylated dolichol-linked oligosaccharides (e.g., Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-PP-Dol or Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-Dol) are the preferred donors for the OST (Turco et al., 1977; Spiro et al., 1979a; Verostek et al., 1993), the most reasonable interpretation of our results is that the novel Hex\textsubscript{7}GlcNAc\textsubscript{2}-NYT product is the triglucosylated compound Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT that would arise by the addition of the α1,6 dimannose antenna and terminal glucose residues to Man\textsubscript{9}GlcNAc\textsubscript{2}-PP-Dol. The glycopeptide products obtained from the OST endpoint assay of the Δalg3 OS-PP-Dol preparation were digested with Endoglycosidase H to provide structural evidence to support this conclusion. Aliquots (2–5 pmol) of the Δalg3 glycopeptides or a Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT standard were digested for 24 h with a large excess of Endo H (500 NEB units). The digestion products were applied to the analytical HPLC column to resolve GlcNAc-NYT from any undigested glycopeptides. Unlike Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT (Hex\textsubscript{5}GlcNAc\textsubscript{2}-NYT) which was quantitatively converted to GlcNAc-NYT by the digestion, Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT and Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-NYT were much less sensitive to digestion.
Endo H digestion (Figure 4C) consistent with the absence of both the α1-3 and α1-6 mannos antenna that confer sensitivity to Endo H (Maley et al., 1989). The Hex$_3$GlcNAc$_2$-NYT product showed intermediate sensitivity to Endo H digestion (Figure 4C) consistent with the presence of the α1-6 mannos antenna.

The dolichol-linked oligosaccharides isolated from ∆alg3 cells were resolved into three peaks by preparative HPLC as detected with the OST endpoint assay (Figure 4D). The glycopeptide products from selected fractions were resolved with respect to oligosaccharide size (Figure 4E) to determine which peak contains each of the major dolichol-linked oligosaccharides. Glycopeptide analysis revealed that Man$_5$GlcNAc$_2$ accounted for 98% of the oligosaccharides in the major peak from the preparative HPLC column (Figure 4E, F-38). Clearly, highly purified Man$_5$GlcNAc$_2$-PP-Dol can be isolated from the ∆alg3 mutant cells in a high yield by this procedure. Although the second peak to elute from the preparative column was enriched in Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol, this peak was contaminated with Man$_5$GlcNAc$_2$-PP-Dol plus the less abundant compounds that contain 6, 7, and 9 hexose units (Figure 4E, F-46). The third peak from the preparative column was enriched in Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol, but contained detectable amounts of Man$_5$GlcNAc$_2$-PP-Dol and Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol (Figure 4E, F-52). The observation that we can obtain Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol in roughly 80% purity by a single chromatographic step is particularly remarkable given that this minor dolichol-linked oligosaccharide had not been detected in previous composition analysis of the Alg3 lipid-linked oligosaccharides (Huffaker and Robbins, 1983; Aebi et al., 1996).

Analysis of OS-PP-Dol preparations by TLC on silica gel plates

The dolichol-linked oligosaccharide preparations isolated from porcine pancreas and ∆alg3 yeast cultures were spotted onto

Fig. 4. Purification of Man$_5$GlcNAc$_2$-PP-Dol from a ∆alg3 yeast culture. (A–C) OST assays containing purified yeast OST (14 fmol), 250 pmol of Nε-Ac-Asn-Tyr-Thr-NH$_2$, and 18 pmol of a ∆alg3 OS-PP-Dol preparation were incubated at 25°C for 30 min or 72 h. (A) Glycopeptide products from the 72-h endpoint assay were eluted from ConA beads and resolved by HPLC to determine the composition of the ∆alg3 OS-PP-Dol preparation. The elution position of the Hex$_{12}$-NYT standards is shown for comparison. (B) The percentage of each dolichol-linked oligosaccharide (Hex$_x$GlcNAc$_y$-PP-Dol to Hex$_x$GlcNAc$_y$-PP-Dol) that was transferred to a peptide acceptor during the 30-min OST assay was determined by HPLC analysis of glycopeptide products. (C) Glycopeptide products from OST endpoint assays containing purified Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol or the ∆alg3 OS-PP-Dol preparation were eluted from the ConA beads, taken to dryness, and suspended in Endo H digestion buffer. Aliquots of the glycopeptides were incubated for 24 h in the presence or absence of Endo H (500 U, New England Biolabs) prior to HPLC chromatography to resolve the undigested glycopeptides from GlcNAc-NYT. The percentage of the Hex$_x$GlcNAc$_y$-NYT, Hex$_x$GlcNAc$_y$-NYT, Hex$_x$GlcNAc$_y$-NYT, and Hex$_x$GlcNAc$_y$-NYT products that were converted to GlcNAc-NYT was calculated from the HPLC profiles. (D) The elution profile of ∆alg3 dolichol-linked oligosaccharides on the preparative aminopropyl silica column was determined with the OST endpoint assay. The concentration gradient of NH$_4$OAc in the eluate fractions is indicated by the dashed line. (E) The OS-PP-Dol composition of selected fractions from the preparative HPLC column in C was determined by HPLC analysis of glycopeptides from the OST endpoint assays. The structure of the major oligosaccharide species for each analysis is depicted.
silica gel thin layer plates and subjected to chromatography in CHCl₃:CH₃OH:H₂O (10:10:3). Organic compounds that were resolved on the TLC plate were visualized with iodine vapor (not shown) or with an anisaldehyde–sulfuric acid spray reagent (Figure 5A). The OS-PP-Dol preparation isolated from the Δalg3 yeast microsomes contains at least seven compounds that can be detected by staining with the anisaldehyde–sulfuric acid spray (Figure 5A, L) and iodine vapor (not shown). The number of compounds that could be detected on the TLC plate was greatest with whole tissues, intermediate with crude microsomes, and least when purified RER were used as the starting material for the OS-PP-Dol isolation (data not shown). The majority of the components in the crude OS-PP-Dol preparation did not bind to the preparative aminopropyl silica column, but were instead recovered in a flow-through fraction (Figure 5A, UB) or in the wash fractions (not shown). The TLC lanes designated as P1, P2, and P3 correspond to the three peak fractions from the column shown in Figure 4D. Similar amounts (~50 pmol) of dolichol-linked oligosaccharide were spotted onto each of the TLC lanes except for the UB fraction, which lacked detectable OS-PP-Dol. Faint spots corresponding to Man₉GlcNAc₂-PP-Dol (P1), Glc₃Man₉GlcNAc₂-PP-Dol (P2), and Glc₃Man-Man₉GlcNAc₂-PP-Dol (P3) were visible in the region of the TLC plate designated by the bracket labeled OS-PP-Dol. More important, the major compounds visible in the crude OS-PP-Dol isolate were absent or were present in greatly reduced amounts (see solvent front in Figure 5A). As reported previously for this TLC system (Badet and Jeanloz, 1988), the mobility of the OS-PP-Dol decreases with increasing saccharide units. The column load fraction (L) but not the flow-through fraction (UB) contains a spot that comigrates with the Man₉GlcNAc₂-PP-Dol spot in the P2 fraction. Additional evidence concerning the mobility of OS-PP-Dol compounds on the TLC system was obtained by probing a TLC plate with ConA-FITC (Figure 5B). The Man₉GlcNAc₂-PP-Dol (M9), Glc₃Man₉GlcNAc₂-PP-Dol (G2), and Glc₃Man-GlcNAc₂-PP-Dol (G3) pools obtained from the preparative HPLC column (Figure 2) were resolved on TLC plates as in Figure 5A. Each of the purified OS-PP-Dol samples migrated as a single spot in the region of the plate designated by the labeled bracket. For unknown reasons, the ConA-FITC detection procedure for locating OS-PP-Dol on TLC plates was less sensitive than anticipated and showed considerable variation between experiments.

**Monosaccharide analysis of purified OS-PP-Dol**

The use of the OST endpoint assay to accurately determine the concentration and composition of OS-PP-Dol samples depends on the irreversibility of the oligosaccharyltransferase reaction, the stability of the donor oligosaccharide substrate and the glycopeptide product, and the efficient conversion of the donor substrate into product. To ensure that these conditions were satisfied, we sought a second independent method that could be used to quantify OS-PP-Dol in our preparations. Samples of the Glc₃Man₉GlcNAc₂-PP-Dol and the Man₉GlcNAc₂-PP-Dol preparations were sent to the Complex Carbohydrate Research Center at the University of Georgia. A known quantity of fucose was added to each sample prior to releasing monosaccharides by acid hydrolysis (2 N trifluoroacetic acid at 100°C for 4 h). The monosaccharide composition was determined by Dionex high pH–anion exchange chromatography (HPAEC) (Hardy and Townsend, 1994) and is presented in Table I. The ratio of monosaccharides released by acid hydrolysis of Glc₃Man₉GlcNAc₂-PP-Dol pool supports our conclusion that the Glc₃Man₉GlcNAc₂-PP-Dol preparation contains only low amounts of incompletely assembled dolichol-linked oligosaccharides. Although monosaccharide analysis of the oligosaccharides derived from the Man₉GlcNAc₂-PP-Dol yielded more glucose than expected, the OS-PP-Dol sample...
submitted for analysis (~225 pmol of OS-PP-Dol) approached the minimal quantity that can be analyzed by this procedure. In addition to the unexpected amount of glucose, traces of GalNAc were also present in the Man$_5$GlcNAc$_2$-PP-Dol-derived sample. One possible explanation for the presence of these two monosaccharides in the Man$_5$GlcNAc$_2$-PP-Dol pool may be contamination with a glycolipid. Inclusion of fucose as an internal standard prior to acid hydrolysis allowed quantification of the OS-PP-Dol based on the recovery of mannose relative to fucose (Table I). The OS-PP-Dol concentrations determined by this approach were in good agreement with the results of the OST endpoint assay, indicating that the latter assay does not underestimate the OS-PP-Dol concentration of a dolichol-linked oligosaccharide sample.

**Discussion**

The method we have developed for the large-scale isolation of dolichol-linked oligosaccharides from vertebrate tissues and fungal cells has several advantages relative to previous procedures. The heterogeneity of tissue dolichol-linked oligosaccharide preparations was substantially reduced by minimizing disruption of intracellular organelles and by reducing the time between tissue collection and organic solvent extraction. Hydrolysis of the terminal glucose residues from dolichol-linked oligosaccharides by ER glucosidases may have contributed to the low percentage of Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol that has been reported for calf pancreas OS-PP-Dol (Badet and Jeanloz, 1988; Gibbs and Coward, 1999). The extensive degradation of the bovine dolichol-linked oligosaccharides that we had obtained with an earlier procedure (Figure 1B) is also reminiscent of that observed by other investigators (Badet and Jeanloz, 1988; Gibbs and Coward, 1999). The degradation products may arise by exposure of the dolichol-linked oligosaccharides to cytoplasmic and lysosomal glucosidases.

We have used a sensitive OST endpoint assay to monitor the elution of dolichol-linked oligosaccharides from the preparative HPLC column. The validity of using the OST endpoint assay to determine the concentration of OS-PP-Dol samples was verified by an independent analytical procedure (Table I). One advantage of this detection method is that the glycopeptide products can be used to determine the concentration and composition of the dolichol-linked oligosaccharides in the eluate fractions. Consequently, we need not metabolically label the dolichol-linked oligosaccharides prior to chromatography. Alternatively, the OS-PP-Dol compounds in the eluate fractions can be detected with a ConA binding assay, as described here, or by the inclusion of $^{3}H$ mannose-labeled dolichol-linked oligosaccharides.

A single chromatography step using the aminopropyl silica HPLC column achieved a remarkable resolution of two dolichol-linked oligosaccharides that differ by one or more sugar residues. To our knowledge, previously described chromatographic methods for resolving individual OS-PP-Dol compounds are either less rapid and less versatile (Sharma et al., 1990), or are primarily applicable to the separation of small quantities of radiolabeled compounds (Wells et al., 1981).

The procedure we have developed for the purification of dolichol-linked oligosaccharides from alg mutant yeast strains will yield pure bona fide intermediates in the assembly pathway. As shown here for the Δalg3 mutant, the steady-state dolichol-linked oligosaccharide pool isolated from an alg mutant yeast culture will likely contain several dolichol-linked oligosaccharide assembly intermediates. The use of substrate preparations that contain other assembly intermediates could complicate the biochemical analysis of a glycosyltransferase or the oligosaccharyltransferase. For example, if total dolichol-linked oligosaccharides isolated from the Δalg3 mutant were used as an assay substrate for the mammalian OST, the initial rates of glycopeptide formation would primarily reflect transfer of Glc$_3$Man$_9$GlcNAc$_2$ and Glc$_3$Man$_9$GlcNAc$_2$, rather than Man$_5$GlcNAc$_2$.

As shown in Figure 6 a number of authentic assembly intermediates could be isolated from cultures of currently available Δalg mutants. The mannosyltransferase responsible for the addition of the terminal α-1,2 linked mannose residue to the α-1,6 dimannose antenna has not been identified. To date, only three yeast glycosyltransferase genes (ALG7, ALG1, and ALG2) have been identified that mediate assembly of Man$_5$GlcNAc$_2$-PP-Dol (Figure 6). The alg1 mutant accumulates GlcNAc$_2$-PP-Dol, and the alg2 mutant accumulates a mixture of Man$_5$GlcNAc$_2$-PP-Dol and Man$_5$GlcNAc$_2$-PP-Dol (Huffaker and Robbins, 1982, 1983; Jackson et al., 1989, 1993). Large-scale isolations of pure dolichol-linked oligosaccharides from the alg1 and alg2 mutants should be feasible using organic solvent extraction procedures that are appropriate for the early assembly intermediates (Huffaker and Robbins, 1982).

As observed previously (Huffaker and Robbins, 1983; Aebi et al., 1996), the most abundant lipid-linked oligosaccharide that is synthesized by an alg3 mutant is Man$_5$GlcNAc$_2$-PP-Dol. This assembly intermediate is a poor substrate for both the α-1,3 glucosyltransferase that is encoded by ALG6 (Reiss et al., 1996) and the α-1,6 mannosyltransferase that is encoded by ALG12 (Burd and Aebi, 1999). It has been proposed that an important specificity determinant for the Alg6p glucosyltransferase is the complete α-1,3,α-1,2 dimannose antenna that cannot be synthesized by the alg3 or alg6 mutants (Aebi et al., 1996; Burda et al., 1996; Cipollo and Trimble, 2000). Support for this view is provided by the observation that a Δalg12 yeast strain assembles a higher proportion of a triglucosylated donor oligosaccharide (Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol) than a Δalg3 strain (Burd and Aebi, 1999). Man$_5$GlcNAc$_2$-PP-Dol, the most abundant dolichol-linked oligosaccharide that accumulates in a Δalg9 strain, is also a poor substrate for the Alg12p mannosyltransferase and the Alg6p glucosyltransferase (Burd et al., 1996; Cipollo and Trimble, 2000).

We have confirmed and extended these observations by analyzing the steady-state pool of dolichol-linked oligosaccharides in a Δalg3 strain. We observed that Man$_5$GlcNAc$_2$-PP-Dol, Glc$_3$Man$_5$GlcNAc$_2$-PP-Dol, and Glc$_3$Man$_5$GlcNAc$_2$-PP-Dol were present in a 12:2.5:1 ratio. Glc$_3$Man$_5$GlcNAc$_2$-PP-Dol was probably not detected in previous studies due to the low abundance of this compound and the more complex N-linked oligosaccharide profile that was obtained from cultures of the slightly leaky alg3 mutant (Verostek et al., 1991, 1993). However, neither Glc$_3$Man$_5$GlcNAc$_2$-PP-Dol nor Glc$_3$Man$_5$GlcNAc$_2$-PP-Dol were detected in a more recent analysis of $^{3}H$ mannose-labeled dolichol-linked oligosaccharides isolated from the Δalg3 strain (Burd and Aebi, 1999). One possible
The specificity of the Alg2p mannosyltransferase (glycosyltransferase that mediates a given saccharide addition is indicated if alg mutants (Abeijon and Hirschberg, 1990). The yeast gene that encodes the biosynthetic labeling of mammalian wild type (Chapman et al., 1979). This diagram has been adapted from Kelleher et al., 1992). HPLC analysis of the glycopeptide products from OST endpoint assays revealed that the OS-PP-Dol preparation obtained by this procedure contained a high percentage of nonglucosylated oligosaccharides. For example, CDG-1d is caused by a defect in the Dol-P-Man-dependent Man₆GlcNAc₂-PP-Dol:α₁,3 mannosyltransferase encoded by the human ALG3 gene (Korner et al., 1999). Composition analysis of dolichol-linked oligosaccharides isolated from CDG cell lines should be feasible using the OST endpoint assay followed by analytical HPLC. Moreover, the biochemical characterization of the enzyme defects would be aided by the availability of purified dolichol-linked oligosaccharides.

**Materials and methods**

**Isolation of dolichol-linked oligosaccharides from pancreas**

In preliminary experiments, dolichol-linked oligosaccharides were isolated from bovine pancreas by a modification of the method of Das and Heath (1980) as described previously (Kelleher et al., 1992). HPLC analysis of the glycopeptide products from OST endpoint assays revealed that the OS-PP-Dol preparation obtained by this procedure contained a high percentage of nonglucosylated oligosaccharides (Figure 1B). The procedure described below was developed to minimize potential hydrolysis of the OS-PP-Dol compounds by endogenous glycosidases.

Sixty grams of freshly excised pancreas was typically chilled to 0°C by immersion in ice-cold homogenization buffer (20 mM Tris–Cl [pH 7.5], 0.25 M sucrose, 50 mM KOAc, 6 mM Mg(OAc)₂, 1 mM EDTA, 1 mM DTT, 1x protease inhibitor mixture [as defined in Kelleher and Gilmore, 1994] and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). For the dolichol-linked oligosaccharide isolation shown in Figure 1C and 1D, the porcine pancreas was initially chilled in situ using ice-cold Krebs-Ringer buffer. The isolation of a crude microsome fraction from the porcine pancreas occurred in a cold room to minimize glycosidase activities. The chilled tissue was minced and then mixed with 1.3 ml of the homogenization buffer per g of tissue. The 136 ml of resuspended tissue was homogenized in several batches using 25 strokes of a Potter-Elvehjem homogenizer as described for the canine pancreas rough microsome preparation (Walter andBlobel, 1983). The homogenate was centrifuged for 10 min at 10,000 × g in a Sorvall SS34 rotor to remove unbroken cells, nuclei, and large organelles. A floating fat layer was removed with a spatula and discarded before the supernatants were collected and pooled.
The supernatant was diluted to 140 ml with homogenization buffer and divided into four 35-ml portions. Two portions were incubated at 37°C for 60 min in glass bottles to permit hydrolysis of terminal glucose residues on the OS-PP-Dol by endogenous RER glucosidases. All four portions were frozen in liquid nitrogen for storage at −80°C. The crude microsomes that were not exposed to the 37°C incubation served as starting material for the isolation of Glc3ManαGlcNAc2-PP-Dol, while the 37°C treated microsomes served as starting material for the isolation of Man3GlcNAc2-PP-Dol.

The four frozen membrane preparations were rapidly thawed in a 37°C water bath, adjusted to 35 ml with cold homogenization buffer if necessary, and transferred into four 150-ml glass round-bottom centrifuge tubes. The membrane preparations were mixed with 50 ml of cold (4°C) CHCl3:CH3OH (3:2) and homogenized with six 10-s bursts of a Brinkman Polytron homogenizer at maximum speed. After homogenization, an additional 65 ml of cold (4°C) CHCl3:CH3OH (3:2) was added to each centrifuge tube and the contents mixed by vigorous shaking. After centrifugation for 15 min at 4°C and 2500 rpm in a Sorvall HS4 swinging bucket rotor, the upper aqueous and lower organic phases were removed by aspiration and discarded. The particulate interface wafers (~0.4 cm thick) were resuspended in 75 ml of room-temperature CHCl3:CH3OH (3:2) containing 1 mM MgCl2 by polytron homogenization as described above and adjusted to 150 ml with the same solvent mixture. Following centrifugation for 15 min at 4°C and 2500 rpm in a Sorvall HS4 swinging bucket rotor, the supernatant was removed and the precipitate was resuspended in 75 ml of CH3OH:4 mM MgCl2 (1:1) by polytron homogenization, diluted to 150 ml with CH3OH:4 mM MgCl2 (1:1) and centrifuged as above at 4°C. The preceding wash with aqueous methanol was repeated a second time. The precipitate was resuspended by polytron homogenization in 75 ml of CHCl3:CH3OH:H2O (10:10:3), diluted to 150 ml with the same solvent mixture, and incubated for 15 min at 22°C prior to centrifugation for 15 min at 22°C and 2500 rpm in a Sorvall HS4 swinging bucket rotor. The supernatant fraction containing the OS-PP-Dol was removed and saved. Each of the precipitates was reextracted at 37°C with 100 ml of CHCl3:CH3OH:H2O (10:10:3). The supernatants from the second extraction were combined with the first extracts, and the solvents were evaporated under a stream of nitrogen in a fume hood. The dried residues were dissolved in CHCl3:CH3OH:H2O (10:10:3) and pooled to obtain one 7.5-ml sample for each of the two crude membrane preparations (± 37°C incubation). The resuspended extracts were transferred to conical glass centrifuge tubes for a 15-min spin at 1000 × g at 22°C to remove any insoluble material. The clarified extracts were transferred to new glass tubes with Teflon caps, adjusted to 7.5 ml CHCl3:CH3OH:H2O (10:10:3) and stored at −20°C. As determined with an OST endpoint assay, each preparation was about 3.7 µmol of OS-PP-Dol per g of tissue.

Dolichol-linked oligosaccharide isolation from yeast

Lipid-linked oligosaccharides were isolated from a crude microsomal membrane fraction (10,000 × g supernatant fraction). Briefly, the αalg 3 mutant (YG248) (Aebi et al., 1996) was grown at 25°C to a density of 2.2 (A600) in 10.5 L of minimal media (-His) supplemented with adenine (40 µg/ml) using a Bioflo 2000 fermenter. The yeast cells were collected by centrifugation, washed once, and disrupted as described previously (Kelleher and Gilmore, 1994) with 210 ml of glass beads in 190 ml of buffer (50 mM TEA-OAc [pH 7.5], 0.25 M sucrose, 50 mM KOAc, 6 mM Mg(OAc)2, 1 mM EDTA, 1 mM DTT, 1x protease inhibitor mixture, and 0.1 M PMSF). After the glass beads were washed by passage through a nylon mesh filter, a 10,000 × g supernatant fraction was prepared as described (Kelleher and Gilmore, 1994) and adjusted to a total volume of 210 ml. Dolichol-linked oligosaccharides were isolated from the 10,000 × g supernatant fraction as described above for the porcine pancreas, with the exception that all six 35-ml aliquots were used for the isolation without the 37°C preincubation.

HPLC of dolichol-linked oligosaccharides

Individual dolichol-linked oligosaccharides in the extracts prepared from vertebrate tissue or yeast cultures were resolved by chromatography on a 24.3-µm aminopropyl silica HPLC column purchased from Varian/Rainin. The column consisted of a 1 × 6 cm guard column (R0080710C5) followed by a 1 × 25 cm preparative column (R00807010G5), both of which were packed with Microsorb aminopropyl silica (5 µ particle, 100 Å pore) in Dynamax hardware. Solvent resistant valves and fittings were used for all connections. Solvent A was CH3CN:CH3OH:H2O (10:10:3), and solvent B was CHCl3:CH3OH:2 M NH4OAc (pH 7.2) (10:10:3). Prior to chromatography, the column was first washed at a flow rate of 4 ml/min with 250 ml of solvent B followed by 250 ml of solvent A. The flow rate of solvent A was reduced to 2 ml/min for an additional 10 min of equilibration. The 4-ml sample applied to the column contained as much as 15 nmol of a crude OS-PP-Dol preparation dissolved in solvent A. The sample was applied at a flow rate of 2 ml/min, and the column washed with solvent A for 10 min at this flow rate before increasing the flow rate to 4 ml/min over a 1-min period. Flow-through and wash fractions of 40 ml each were collected during a 60-min period after sample injection. Less than 0.2% of the dolichol-linked oligosaccharide was recovered in the flow-through and wash fractions. At the 60-min time point, a 320-ml linear gradient of 0–40% solvent B was initiated at a flow rate of 4 ml/min. This gradient corresponds to an increase of 1.3 mM NH4OAc per 4-ml fraction of eluate. At the 140-min time point, a 1-min gradient between 40% and 100% B was followed by a 15-min wash with 100% B.

Oligosaccharyltransferase endpoint assay

The yeast OST was purified by a modification of a previously described procedure (Kelleher and Gilmore, 1994), using a strain expressing a hexahistidine-tagged OST1 subunit. The strain construction and purification procedure will be described elsewhere (Karaoglou, Kelleher, and Gilmore; manuscript in preparation). The iodinated tripeptide acceptor Nα-Ac-Asn-[125I]Tyr-Thr-NH2 (15,000–25,000 cpm/nmol) was prepared as described previously (Kelleher et al., 1992). The 50 µl endpoint assays to estimate OS-PP-Dol concentration and to yield products for composition analysis contained 10–15 fmol of purified yeast OST, 5 µM Nα-Ac-Asn-[125I]Tyr-Thr-NH2, approximately 2.5–18 pmol of OS-PP-Dol, 50 nM Tris-Cl (pH 7.4), 2.5 mM NaCl, 2 mM MnCl2, 3 mM MgCl2, 0.006% digitonin, 1.35 mM phosphatidylcholine,
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1 mM DTT, 15% DMSO, 5% glycerol, and 0.13 mM EDTA. For more precise composition analysis, the OST endpoint assays were supplemented with 1.3 mM each of deoxy-
oxorijirimycin and deoxyxamnajirimycin. The OST endpoint assays were terminated after 48–72 h by the addition of 0.1 ml of ice-cold 9.4% Nikkol (octaethylenglycol-mono-N-dodecyl ether; Nikko Chemicals, Inc.), 11.3 mM EDTA. The assay was diluted with 1 ml of ice-cold 50 mM Tris–Cl (pH 6.7), 1 M NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂, 0.3% Nikkol, and the glycopeptide products collected during a 20-min end-
derway incubation with 100 µl of a 1:1 suspension of ConA Sepharose beads equilibrated in ConA-HS buffer (50 mM Tris–Cl [pH 6.7], 1 M NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂, 0.02% Nikkol). The beads were collected by centrifugation and washed four times with 1 ml of Con A-HS buffer. Glycopeptide products were quantified by gamma counting and corrected for nonspecific binding of Nα-Ac-
Asn-[125I]Tyr-Thr-NH₂ to the ConA beads in control assays that lacked either enzyme or OS-PP-Dol.

HPLC analysis of glycopeptides

For oligosaccharide composition analysis, the ConA Sepharose beads were washed twice with 1.4 ml of cold water, after which the glycopeptides were eluted by two rapid extractions at 25°C with 0.75 ml of 60% acetonitrile, 0.2 N HCl. The combined eluates were dried in a Speed-Vac and resuspended in 300 µl of 70% acetonitrile, 3% acetic acid adjusted to pH 5.5 with triethylamine (buffer A). The HPLC conditions used for resolving neutral glycopeptides on the basis of oligosaccharide size were based on the method of Mellis and Baenziger (1983). The HPLC column was a Varian/Rainin aminopropyl silica column composed of a 0.46 × 6 cm guard column (R0080700G5) followed by a 0.46 × 25 cm analytical column (R0080725SC5), both of which were packed with Microsorb aminopropyl silica (5 µm particle, 100 Å pore). Buffer B for the HPLC column was 3% acetic acid in H₂O adjusted to pH 5.5 with triethylamine. The concentration of triethylamine in buffers A and B is about 90 mM and 432 mM, respectively. Samples were injected into the column at a flow rate of 1 ml/min, followed by a 10-min wash with buffer A. The glycopeptides were eluted with a 60-min linear gradient between 0% and 40% buffer B at a flow rate of 1 ml/min. One hundred 0.5-ml fractions were collected and the quantity of radiolabeled glycopeptide determined by gamma counting. Between chromatography runs, the column was washed with 15 ml of buffer B.

Solid state ConA binding assay

Unlabeled OS-PP-Dol in HPLC fractions was detected using a semiquantitative ConA binding assay. Known quantities of OS-PP-Dol (1–7 pmol) in CHCl₃:CH₃OH:H₂O (10:10:3) or 100-µl aliquots of eluate fractions from the aminopropyl silica column were dried in 70% acetonitrile, 3% acetic acid adjusted to pH 5.5 with triethylamine (buffer A). The assay was performed with a freshly prepared solution containing 1 ml of 4-methoxy-benzaldehyde (Sigma A-0519), 100 ml of glacial acetic acid, and 2 ml of sulfuric acid (Lewis and Smith, 1972). The TLC was baked in an oven at 105°C for 10–120 s at 37°C. A 30-s exposure allowed detection of 1 pmol of OS-PP-Dol per tube. The assay relies on the binding of the OS-PP-Dol to the glass and the insolubility of OS-PP-Dol in aqueous solutions lacking detergent and is not affected by the presence of 200 mM NH₄OAc in the assay sample.

TLC of dolichol-linked oligosaccharide

Water-resistant 20 × 20 cm silica thin-layer plates with a 0.2 mm coating on an aluminum support (EM-16487-1) were purchased from VWR. The TLC plates were prerun in a TLC tank that had been preequilibrated with CHCl₃:CH₃OH:H₂O (10:10:3) and dried. Samples from the preparative HPLC columns containing 50–150 pmol of OS-PP-Dol in CHCl₃:CH₃OH:0–0.1 M NH₄OAc (10:10:3) were dried in a Savant Speed-Vac, redissolved in ~15 µl of CHCl₃:CH₃OH:H₂O (10:10:3) and spotted 2.5 cm from the bottom of a TLC plate. The TLC plate was developed in CHCl₃:CH₃OH:H₂O (10:10:3) until the solvent front had risen 11–12 cm from the origin (~60–80 min).

Glucose and mannose containing compounds on the TLC plate were detected with a fluorescein isothiocyanate (FITC) derivative of concanavalin A (Sigma, C-7642). The TLC plates were dried and placed in a polypropylene dish containing 50 ml of a 1% solution of ConA-MS buffer that contained 0.5 µg of ConA-FITC. After 80 min of incubation at 25°C, excess ConA was removed by five successive 10-min washes of the TLC plate with 150 ml of ConA-MS buffer. After excess moisture was removed from the TLC plate by placing it on filter paper, the plate was exposed to broad band UV illumination (290–365 nm) within a Fluoro-S Multi-Imager. The emitted light was collected for 30–180 s after passage through a 530DF60 band pass filter (495–555 nm). The images were exported from the BioRad MultiAnalyst software to Adobe Photoshop. When the ConA detection method for TLC plates worked well, the TLC could be incubated for 48 h in ConA buffer-MS without signal loss. The unexpectedly low (~75 pmol of OS-PP-Dol) and somewhat variable sensitivity of this detection method suggests that the TLC plate may interfere with ConA detection of OS-PP-Dol or that the OS-PP-Dol may either hydrolyze during chromatography or incubation in the ConA-MS buffer. The TLC plates not exposed to ConA-FITC were stained with anisaldehyde to visualize as many compounds as possible in the crude OS-PP-Dol preparation. Preliminary experiments showed that no additional compounds were detected by UV absorption of F254-impregnated silica gel plates or by an iodine vapor staining, alone or in combination with an anisaldehyde stain. The dried TLCs were sprayed until saturated with a freshly prepared solution containing 1 ml of 4-methoxy-benzaldehyde (Sigma A-0519), 100 ml of glacial acetic acid, and 2 ml of sulfuric acid (Lewis and Smith, 1972). The TLC was baked in an oven at 105°C for 45–60 min to allow color.
development. The images were captured using Microtek Scanmaker III and imported into Adobe Photoshop where they were converted into grayscale images for publication. 50 pmol of OS-PP-Dol could be detected by anisaldehyde staining of TLC plates.

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Abbreviations
ALG, asparagine-linked glycosylation; ConA-FTTC, Concanavalin A labeled with fluorescein isothiocyanate; HPLC, high-pressure liquid chromatography; HPAEC, high pH-anion exchange chromatography; OST, oligosaccharyltransferase, OS-PP-Dol, dolichol-linked oligosaccharide; RER, rough endoplasmic reticulum; TLC, thin-layer chromatography.

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