# Specific transbilayer translocation of dolichol-linked oligosaccharides by an endoplasmic reticulum flippase

**Sumana Sanyal and Anant K. Menon[1]**

Department of Biochemistry, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065

Edited by Phillips W. Robbins, Boston Medical Center, Boston, MA, and approved November 21, 2008 (received for review October 10, 2008)

**The oligosaccharide donor for protein *N*-glycosylation, $Glc_3Man_9GlcNAc_2$-PP-dolichol, is synthesized via a multistep pathway that starts on the cytoplasmic face of the endoplasmic reticulum (ER) and ends in the lumen where the glycosylation reaction occurs. This necessitates transbilayer translocation or flipping of the lipid intermediate $Man_5GlcNAc_2$-PP-dolichol (M5-DLO) across the ER membrane. The mechanism by which M5-DLO—or any other lipid—is flipped across the ER is unknown, except that specific transport proteins or flippases are required. We recently demonstrated M5-DLO flipping activity in proteoliposomes reconstituted from detergent-solubilized ER membrane proteins and showed that it was ATP-independent and required a trypsin-sensitive protein that sedimented at approximately 4S. By using an activity-enriched fraction devoid of glycerophospholipid flippase activity, we now report that M5-DLO is rapidly flipped in the reconstituted system with a time constant $\tau$ <2 min, whereas its triantennary structural isomer is flipped slowly with $\tau$ >200 min. DLOs larger than M5-DLO are also poorly translocated, with $\tau$ ranging from approximately 10 min to >200 min. We conclude that (i) the number and arrangement of mannoses in the DLO glycan has a profound effect on the ability of the DLO to be translocated by the flippase, (ii) glycan size *per se* does not dictate whether a DLO will be flipped, and (iii) the flippase is highly specific for M5-DLO. Our results suggest a simple structural model for the interaction between the DLO head group and the flippase.**

N-glycosylation | Rft1 | proteoliposome

The majority of proteins that enter the eukaryotic secretory pathway are *N*-glycosylated by oligosaccharyltransferase (OST) as they emerge from the protein translocon into the lumen of the endoplasmic reticulum (ER). OST recognizes glycosylation sequons (Asn-X-Ser/Thr motifs) in the nascent polypeptide and transfers a triantennary tetradecasaccharide from the glycolipid $Glc_3Man_9GlcNAc_2$-PP-dolichol (Fig. 1*A*) to the side-chain amide of the asparagine residue in the sequons.

For *N*-glycosylation to take place, $Glc_3Man_9GlcNAc_2$-PP-dolichol must be available in the ER lumen. Biosynthesis of this lipid involves the stepwise addition of components to dolichol phosphate and occurs in two distinct phases (1–4). The first seven reactions take place on the cytoplasmic face of the ER and use the soluble sugar donors UDP-GlcNAc and GDP-Man to generate the key intermediate $Man_5GlcNAc_2$-PP-dolichol (M5-DLO; structure within the dotted lines in Fig. 1*B*). M5-DLO is then translocated or flipped across the bilayer into the ER lumen where lumenally oriented glycosyltransferases add the next seven sugars. These sugars are derived from the glycolipids Man-P-dolichol and Glc-P-dolichol, both of which are synthesized on the cytoplasmic face of the ER and must be flipped into the ER lumen to participate in the glycosyltransfer reactions that convert M5-DLO to $Glc_3Man_9GlcNAc_2$-PP-dolichol. Thus three different lipids must be flipped across the ER membrane to synthesize the oligosaccharide donor for protein *N*-glycosylation. Lipid flipping across the ER is also important in the biosynthesis of glycosylphosphatidylinositols (GPIs) and glycosphingolipids (5, 6), as well as in the biogenesis of the ER where transbilayer movement of phosphatidylcholine (PC) and other "membrane-building" phospholipids is key to the uniform expansion of the ER membrane during growth (7–11).

Whereas the in-plane rotational and translational diffusion of phospholipids is rapid, the transbilayer translocation of phospholipids across synthetic membranes is extremely slow. The energy barrier that must be overcome to move the polar headgroup of the lipid through the hydrophobic interior of the bilayer ranges from approximately 20 kcal/mol for PC to >130 kcal/mol for M5-DLO (2, 12, 13). The ER has lipid transport proteins or flippases that increase the rate of lipid flipping to a physiologically relevant time-scale. These proteins are required to flip M5-DLO, Man-P-dolichol, and Glc-P-dolichol, as well as glycerophospholipids, GPIs, and glucosylceramide. Because different flippases would be needed to transport these structurally distinct lipids, the ER likely possesses a family of these proteins comprising five to six members. No ER flippase has been identified.

We recently demonstrated M5-DLO flippase activity in proteoliposomes reconstituted from detergent-solubilized ER membrane proteins (14, 15). The activity could be resolved from the genetically identified flippase candidate Rft1 (14, 16), as well as from the previously described ER glycerophospholipid flippase activity (10, 17–19) by velocity sedimentation and dye resin chromatography of the detergent extract before reconstitution (14, 15). Flipping of both M5-DLO and glycerophospholipids was ATP-independent. We now report, that in contrast to the glycerophospholipid flippase which is relatively unspecific and capable of transporting a variety of glycerophospholipids (19, 20), the M5-DLO flippase is highly specific. By using a fraction enriched in M5-DLO flippase activity and devoid of glycerophospholipid flippase activity, we show that DLOs with higher order glycan head groups are transported more slowly than M5-DLO by more than an order of magnitude, and that the transport of nonnatural isomers, including a structural isomer of the physiological M5-DLO substrate, is negligible. Our data are consistent with a simple structural model for the interaction between the DLO head group and the flippase (Fig. 1*B*).

## Results

**M5-DLO Flippase Activity in Proteoliposomes Reconstituted from a Detergent Extract of Rat Liver ER Membrane Proteins.** Mannosylated DLOs dissolve readily in a mixture of chloroform, methanol, and water, but are insoluble when bound to the lectin Concanavalin A (Con A) (14, 15, 21). We used this property of DLOs as the basis for a flippase assay (Fig. 2*A*). When M5-DLO-containing large unilamellar liposomes are treated with Con A, approximately 50% of the M5-DLOs are extracted by solvent (Fig. 2*A*

Author contributions: S.S. and A.K.M. designed research; S.S. performed research; S.S. and A.K.M. analyzed data; and S.S. and A.K.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: akm2003@med.cornell.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0810225106/DCSupplemental.



BIOCHEMISTRY

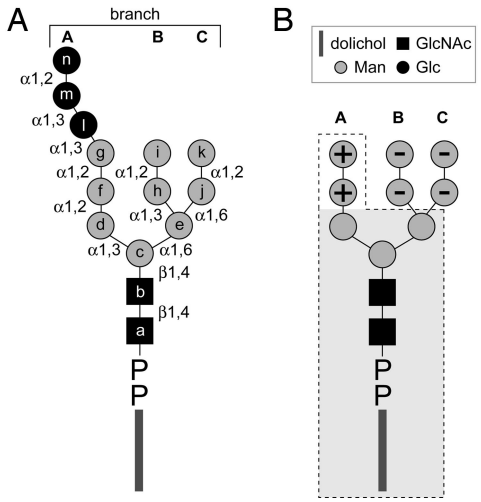


**Fig. 1.** Dolichol-linked oligosaccharides. (*A*) Structure of the oligosaccharide precursor of protein *N*-glycans. The oligosaccharide is synthesized in the ER by stepwise addition of components to dolichol-phosphate. The order of component addition is indicated (a–n). Mannose residues c–g are derived directly from GDP-mannose on the cytoplasmic face of the ER, whereas residues h–k are derived from Man-P-dolichol in the ER lumen. The three branches of the triantennary structure are annotated A, B, and C. Symbols are indicated at the top of *B*. (*B*) The structure of M5-DLO, the physiological substrate for the DLO flippase, is enclosed within the dashed lines. Our results indicate that M3-DLO (shaded box) is flipped almost as well as M5-DLO, and that the A-branch mannose residues indicated by ''+'' enhance flipping whereas those indicated by ''-'' reduce the rate of flipping.

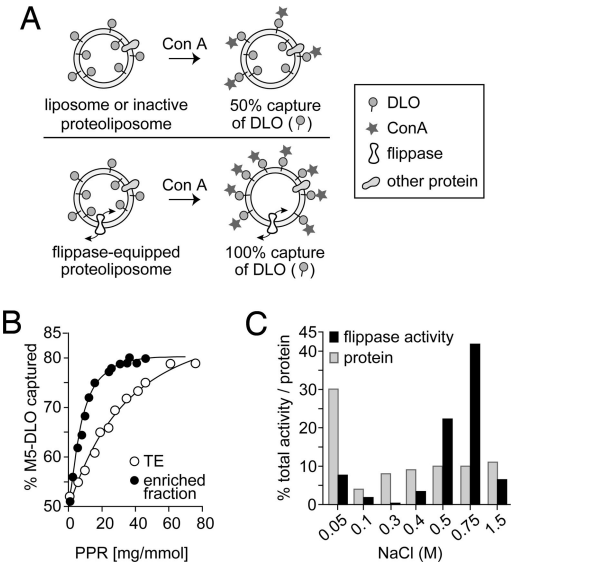


**Fig. 2.** Flippase assay and isolation of a DLO-flippase-enriched fraction. (*A*) Flippase assay based on capture of surface-exposed DLOs by Con A. See *Results* for details. (*B*) M5-DLO flipping in proteoliposomes. Different amounts of TE (○) or a TE-derived fraction enriched in DLO flippase activity (obtained as in *C*). (●) were reconstituted with egg PC and [$^3$H]M5-DLO to generate vesicles with protein/phospholipid ratios (PPR) ranging from 0–100 mg protein/mmol phospholipid. The samples were incubated on ice with Con A for 30 min and processed to determine the fraction of [$^3$H]M5-DLO captured by Con A. (*C*) Fractionation of TE on Cibacron Blue dye resin. TE was loaded onto Cibacron blue dye resin, and the bound material was eluted in six sequential steps with salt. The 0.05-M step corresponds to unbound (flow-through) material. Fractions eluted from the resin were reconstituted and assayed for M5-DLO flippase activity. The percentage of activity and protein recovered (relative to the load) is indicated. The specific activity of M5-DLO flippase activity in the fractions eluted with 0.5 and 0.75 M NaCl was 3.2 and 4.2 (relative to unfractionated TE), respectively. The data are representative of six independent experiments.

*Top*). This fraction corresponds to molecules located in the inner leaflet of the liposomes because M5-DLOs in the outer leaflet bind Con A and resist extraction. In proteoliposomes with M5-DLO flippase activity, M5-DLOs in the inner leaflet are translocated to the outer leaflet where they can be captured by Con A (Fig. 2*A Bottom*). This will result in approximately 100% of the M5-DLOs becoming resistant to solvent extraction. For a mixed population of vesicles in which some vesicles contain a flippase whereas others do not, the percentage of M5-DLO that is captured by Con A and rendered resistant to solvent extraction will range between 50 and 100%, reflecting the proportion of flippase-containing vesicles in the ensemble.

We used Triton X-100-solubilized rat liver, ER-membrane proteins (Triton extract; TE) as a source of M5-DLO flippase activity. Proteoliposomes were reconstituted from egg phosphatidylcholine (ePC), trace quantities of [$^3$H]M5-DLO and TE. When these vesicles were incubated with Con A for 30 min (an end-point measurement for M5-DLO flipping; see below), the percentage of [$^3$H]M5-DLO captured was greater than the approximately 50% value obtained with protein-free liposomes. Capture increased as a function of the protein/phospholipid ratio (PPR) of the preparation, reaching a plateau of approximately 80% in vesicle populations with a PPR of approximately 60 mg/mmol (Fig. 2*B*, open circles). The abundance of the M5-DLO flippase may be deduced from the initial slope of the plot to be approximately 0.5% by weight of proteins in the TE (10, 15). These results extend our recent report of the reconstitution of M5-DLO flippase activity in proteoliposomes containing yeast microsomal proteins (15).

**Chromatography on Cibacron Blue Dye Resin Yields a Fraction Enriched in M5-DLO Flippase Activity.** To generate a flippase-enriched fraction for further study, we chromatographed TE on Cibacron blue dye resin. The extract was incubated with resin and bound proteins were eluted in steps with progressively increasing salt concentrations. Silver-stained SDS/PAGE gels revealed that each of the fractions had a different protein composition, indicating successful separation of TE proteins (data not shown). Fractions were reconstituted into proteoliposomes and assayed for M5-DLO flippase as well as glycerophospholipid flippase activities; the latter was assayed by using a fluorescent analog of phosphatidylcholine (PC) as previously described (17, 19). Both activities bound quantitatively to the dye resin. The majority of M5-DLO flippase activity was eluted with 500 and 750 mM NaCl (Fig. 2*C*) whereas glycerophospholipid flippase activity eluted at a lower salt concentration (300–400 mM; data not shown). These data indicate that M5-DLO and glycerophospholipid flipping are independent activities because of distinct proteins. To measure the fold enrichment of M5-DLO flippase activity precisely, we generated a dose-response plot by reconstituting different amounts of the 750 mM salt-eluted material (Fig. 2*B*, filled circles). From the initial slope of the plot we determined that the 750 mM salt-eluted fraction was approximately 4-fold enriched in M5-DLO flippase activity relative to TE. All further experiments were performed with this enriched fraction.

**Comparison of Biosynthetic DLOs and Their Structural Isomers Reveals that the DLO Flippase Is Highly Specific for M5-DLO.** To define the specificity of the DLO flippase, we prepared a series of radiolabeled DLOs (Fig. 3*A*) for testing in the reconstitution assay. We chose intermediates in the DLO biosynthetic pathway, as well as nonnatural structures such as iM5-DLO, a triantennary structural isomer of M5-DLO. We obtained M3-, M5-, M6-, M7-, iM7-, M9-, and G2M9-DLO from well-characterized yeast glycosylation mutants that had been metabolically labeled with

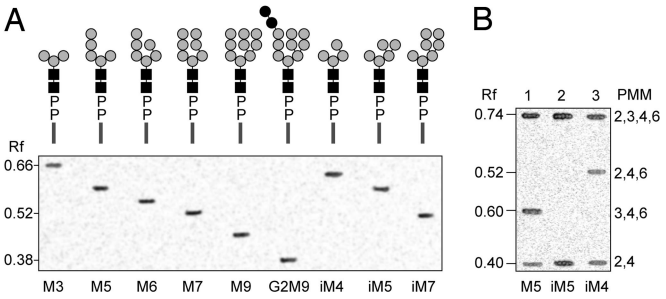


**Fig. 3.** Dolichol-linked oligosaccharides used to assay flippase specificity. (*A*) Schematic structures of DLOs isolated from yeast glycosylation mutants, or generated from M7- or M9-DLO by treatment with *T. reesei* 1,2-α-D-mannosidase (iM4-, iM5-DLO). Analysis of [$^3$H]DLOs (≈3,000 cpm/lane) by TLC; the chromatogram was exposed to a tritium screen and visualized by phosphorimager. Relative migration (Rf) of the DLOs is indicated on the left. (*B*) Methylation linkage analysis of the headgroups of [$^3$H]mannose-labeled M5-, iM5-, and iM4-DLO. Partially methylated [$^3$H]mannose (PMM) residues recovered from the analysis were resolved by TLC and visualized by phosphorimager. The identity of individual PMM residues (2,3,4,6-tetramethyl mannose, 3,4,6- and 2,4,6-trimethyl mannose, and 2,4-dimethyl mannose) is indicated on the right. Relative migration (Rf) is indicated on the left.

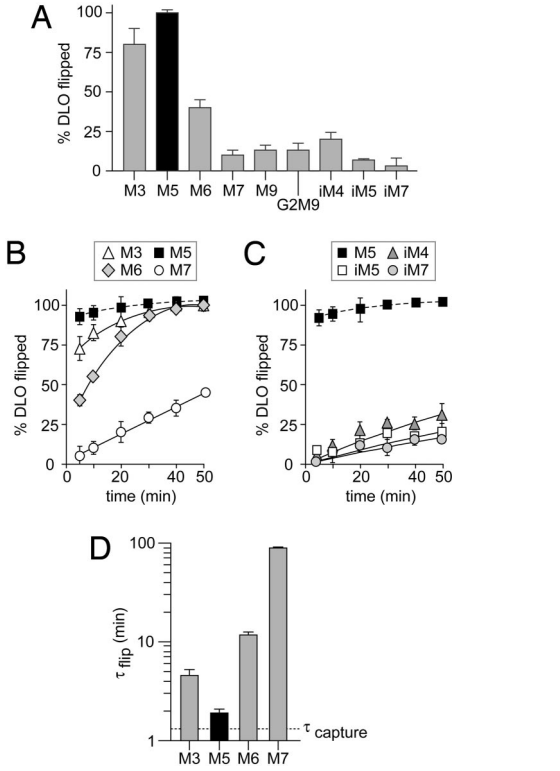


**Fig. 4.** Specificity of the M5-DLO flippase. (*A*) [$^3$H]DLOs were reconstituted with egg PC and a flippase activity-enriched fraction (see Fig. 2) at a PPR of approximately 10 mg/mmol. Protein-free liposomes were prepared in parallel. Vesicles were incubated with Con A on ice for 20 min and the percentage of [$^3$H]DLO that was captured by Con A was determined. Data were corrected for the percentage of [$^3$H]DLO that was captured in liposomes (50 ± 2% for all DLOs) and normalized to the maximum extent of capture obtained with [$^3$H]M5-DLO ≈70%, as expected for vesicles with PPR ≈10 mg/mmol (Fig. 2*B*)]. (*B*) Kinetics of [$^3$H]M3-, M5-, M6-, and M7-DLO flipping at 25 °C were determined for samples prepared as described in panel *A*. The earliest time point analyzed was 5 min to allow complete binding of Con A to DLOs. Data were normalized as described in panel *A* to generate dimensionless plots as shown. The lines represent mono-exponential fits (see *SI Text*). (*C*) Kinetics of [$^3$H]iM4-, iM5-, and iM7-DLO flipping at 25 °C. Data were obtained as in *B*. (*D*) Time constants ($\tau_{flip}$) (plotted on a logarithmic scale) corresponding to the data shown in *B*; $\tau_{capture}$ represents the kinetics of capture of detergent-solubilized DLOs by Con A. The $\tau_{flip}$ values for M5-DLO and M3-DLO are overestimates because they cannot be adequately resolved from $\tau_{capture}$. Data in *A*–*D* are reported as the mean ± standard deviation of at least two independent experiments.

[2-$^3$H]mannose; M3-, iM4-, and iM5-DLO were obtained by treating [$^3$H]mannose-labeled M5-, M7-, and M9-DLO, respectively, with *T. reesei* 1,2-α-D-mannosidase (22), an exoglycosidase specific for α1,2 mannose linkages. TLC analysis of the DLOs is shown in Fig. 2*A*, demonstrating the purity of the preparations and a relative migration pattern consistent with their expected structures. All of the DLOs bound Con A rapidly and efficiently, and when bound to the lectin became similarly resistant to organic solvent extraction. This observation confirms that the entire spectrum of DLOs shown in Fig. 3*A* can be used in the Con A-based flippase assay.

Although the structure of the DLOs we prepared may be reliably inferred from the mutant strain and specific mannosidase used for their preparation, we carried out additional analyses to confirm their identity. The oligosaccharide head group of the DLOs was released by mild acid hydrolysis and analyzed by HPLC using an amino-bonded column (23); all of the head groups tested (M3, M5, M6, M7, iM4, iM5, and iM7) chromatographed as single peaks (data not shown) with retention times consistent with the structural assignments shown in Fig. 3*A*. Because the TLC and HPLC methods used do not distinguish isomers from the corresponding biosynthetic structures we also analyzed certain head groups by methylation linkage analysis. Figure 3*B* shows such an analysis for the key M5 head group, as well as for the head groups of iM5-DLO and iM4-DLO. Permethylation and glycosidic bond hydrolysis of the M5 head group yielded 2,3,4,6-tetra-, 3,4,6-tri-, and 2,4-dimethyl mannose species as predicted (Fig. 3*B*, lane 1). The iM5 structure contains terminal and doubly branched mannose residues; methylation linkage analysis is predicted to yield tetra- and dimethyl-species, and no trimethylmannose residues. This is indeed what we observed (Fig. 3*B*, lane 2). The iM4 head group is predicted to yield three partially methylated mannose species on methylation linkage analysis. One of these is a 2,4,6-trimethylmannose species not found in the analysis of M5 and iM5. Analysis of the iM4 headgroup confirmed the expected structure (Fig. 3*B*, lane 3). Methylation linkage analyses of the head groups released from M3-DLO and iM7-DLO also yielded the expected pattern of partially methylated mannose species (data not shown). These data confirm the DLO structures shown in Fig. 3*A*.

In an initial survey of flippase specificity, the nine DLO species that we prepared (Fig. 3*A*) were separately reconstituted into proteoliposomes generated from the flippase-enriched fraction described in Fig. 2*B*. The vesicles were incubated with Con A for 20 min on ice and processed to determine the amount of the DLO that was captured by the lectin. The 20-min time point was chosen to discriminate between DLOs that are flipped up to an order of magnitude more slowly than M5-DLO (flipping of M5-DLO is >90% complete within 5 min on ice), and those that may be considered not to be substrates for the flippase. The data (Fig. 4*A*) show that whereas M3-DLO is flipped almost as well as M5-DLO, with transport reaching approximately 80% completion in 20 min, DLOs with higher order glycans (M7-, M9-, and G2M9-DLO), as well as the isomeric structures (iM4-, iM5-, and iM7-DLO) are poor substrates for the flippase. M6-DLO displayed intermediate behavior; flipping was approximately 40% complete after 20 min. Similar data were obtained by using proteoliposomes reconstituted with yeast ER membrane proteins (data not shown) (15). These results indicate that the reconstituted flippase activity is highly selective. The key observation that iM5-DLO, a structural isomer of M5-DLO, is not

BIOCHEMISTRY

flipped (<10% after 20 min compared with 100% for biosynthetic M5-DLO) reinforces this point.

We next assayed transport kinetics at 25 °C anticipating that the rate of flipping of some of the poorer substrates would increase, allowing for better quantitation of their transport. Measurements were done with preparations where each vesicle in the ensemble would be expected to have no more than one flippase. Data were base-line corrected by subtracting the percentage of DLO captured by Con A in liposomes, and normalizing to the maximum extent of flipping to generate a dimensionless readout. Time courses are shown in Fig. 4 *B* and *C*, and time constants ($\tau$) derived from mono-exponential fits of the data in Fig. 4*B* are shown on a logarithmic scale in Fig. 4*D*.

M5-DLO flipping was rapid, with a $\tau$ value of <2 min. Because Con A binds to detergent-solubilized M5-DLO with a $\tau_{capture}$ of approximately 1.3 ± 0.1 min (dotted line in Fig. 4*D*), we conclude that M5-DLO flipping occurs at a rate similar to or greater than the rate at which it is captured by Con A. The rate of M3-DLO flipping ($\tau \approx 4.1 \pm 0.6$ min) was slightly less than that observed for M5-DLO. Although $\tau_{flip}$ for M3-DLO is too close to $\tau_{capture}$ to provide a reliable estimate of flipping rate by using a simple kinetic model, the point that M3-DLO is flipped somewhat more slowly than M5-DLO can be easily seen at the 5-min time point (Fig. 4*B*) where flipping of M3-DLO is approximately 75% complete, compared with approximately 90% for M5-DLO. M6- and M7-DLO were flipped considerably more slowly than M5-DLO, with $\tau_{flip}$ values one and two orders of magnitude greater (≈11.8 ± 0.8 and ≈89.5 ± 2.3 min) for M6-DLO and M7-DLO, respectively. The rate of flipping of all of the isomeric structures tested (iM4-, iM5, and iM7-DLO) was extremely low, making quantitation of $\tau_{flip}$ unreliable (rough estimates for $\tau_{flip}$ are approximately 130 min for iM4-DLO, and >200 min for iM5- and iM7-DLO). We conclude that the DLO flippase is highly specific and is able to discriminate sharply between M5-DLO and other structurally related glycolipids that differ from M5-DLO by as little as a single mannose residue.

## Discussion

Our data suggest a simple correlation between glycan structure and the rate at which a particular mannosylated DLO is translocated by the flippase: mannose residues in the A-branch [indicated with "+" signs (Fig. 1*B*)] promote transport, whereas those in the B- and C-branches [indicated with "−" signs (Fig. 1*B*)] inhibit transport. Of all of the DLOs tested, M5-DLO—the physiological substrate—was the most rapidly translocated, consistent with the model. Removal of mannose residues from the A-branch of M5-DLO and/or elaboration of the B- and C-branches yielded structures that were flipped more slowly. Thus the iM4-, iM5- and iM7-DLO isomers that lack A-branch residues, but possess different numbers of mannoses on the B- and C-branches were poorly flipped, if at all (Fig. 4*C*), and structures such as M6-DLO and M7-DLO that contain a fully mannosylated A-branch and a partial or complete B-branch were flipped 1–2 orders of magnitude more slowly than M5-DLO (Fig. 4 *B* and *C*). For the particular case of M3-DLO, a structure that lacks both the positive contribution of the A-branch mannoses and the negative contribution of the B- and C-branch mannoses, the rate of flipping was only slightly reduced relative to M5-DLO.

The structural correlation we propose suggests that the flippase has a binding site that recognizes the core M3 structure (shaded region, Fig. 1*B*); it is likely that a smaller structural element such as $GlcNAc_2$-PP-dolichol would also interact with the binding site, albeit not as well (see below). Mannose residues in the B- and C-branches would be excluded from the binding site leading to an unfavorable situation in which they could be exposed to the hydrophobic interior of the bilayer. This would slow entry of the headgroup into the bilayer milieu and reduce the rate of transbilayer transit. The unfavorable influence of the B- and C-branches can be offset by the binding interaction between the flippase and the A-branch because, for example, M7-DLO is transported more rapidly than iM7-DLO ($\tau_{flip} \approx 90$ min and >200 min for M7-DLO and iM7-DLO, respectively). It is interesting to speculate that substitution of the free hydroxyl groups on the B- and C- branches with a nonpolar moiety would reduce the energy penalty and make the corresponding synthetic DLOs acceptable flippase substrates. The identification of transport-competent structures via the reconstitution approach described here raises the possibility of future affinity-based approaches for flippase purification.

Because Con A binds poorly to glycans with fewer than three mannose residues (24), the smallest lipid that we could test in our assay was M3-DLO. It is likely that M2-DLO, M1-DLO and $GlcNAc_2$-PP-dolichol would also be flipped by the DLO flippase, because *in vivo* data indicate that the corresponding glycan structures are found on glycoproteins in yeast *alg1* and *alg2* mutants that are defective in the first two mannosylation reactions of DLO assembly (25, 26), and experiments with mammalian microsomes show that $GlcNAc_2$ can be transferred from *in situ* synthesized $GlcNAc_2$-PP-dolichol to proteins (27–29). We hypothesize that the rate at which lower order DLOs are flipped in the reconstituted system would be similar to or lower than that of M3-DLO flipping. Indeed, the flippase may not have to discriminate between lower order DLOs *in vivo* because these lipids do not accumulate significantly unless activated sugar donors are scarce or, whether as suggested below, their synthesis is compartmentalized.

The yeast *alg11* mutant cannot convert M3- to M5-DLO, but is able to elaborate M3-DLO to iM7-DLO in the ER lumen through the action of Man-P-dolichol-dependent glycosyltransferases (30). This indicates that M3-DLO is a substrate for the flippase *in vivo*. Even though iM7-DLO is used as an oligosaccharide donor by OST, Δ*alg11* cells under-glycosylate proteins and display a severe growth defect (16, 30). This phenotype has been attributed to inefficient flipping of M3-DLO, resulting in a reduced rate of iM7-DLO production and inadequate synthesis of glycoproteins. However, because our reconstitution data indicate that M3-DLO is flipped rapidly, almost as well as M5-DLO, we speculate that the inefficient translocation of M3-DLO *in vivo* is because of factors other than a low flipping rate. We previously speculated that early steps of DLO biosynthesis may be laterally compartmentalized within the ER, and that the ability of DLOs to access the flippase is controlled by the ER membrane protein Rft1 (14, 15). Consistent with this interpretation, over-expression of Rft1 in Δ*alg11* cells increased the yield of iM7-DLO and mitigated the growth defect of the cells (16). Thus regulated access of M3-DLO to the flippase—rather than the flipping step itself—may control the rate at which M3-DLO is converted to iM7-DLO in the ER lumen of Δ*alg11* cells.

It is possible that all lower order DLOs have reduced access to the flippase, so that of all of the cytoplasmically synthesized DLO structures only M5-DLO is moved efficiently into the ER lumen. This would ensure synthesis of glucosylated DLOs [structures smaller than M5-DLO do not have a fully developed A-branch and cannot be glucosylated (Fig. 1*A*)] that are better substrates for OST than their nonglucosylated counterparts (31). Regulation at this level would be necessary only on the cytoplasmic face of the ER because higher-order DLOs synthesized in the ER lumen are intrinsically poor transport substrates. Indeed, the inability of the flippase to translocate higher order DLOs efficiently may help to maintain the biosynthetic flux through the pathway by preventing losses because of flipping of DLOs in the reverse direction.

The contrast between the sharp specificity of the M5-DLO flippase and the relatively unspecific character of the ER glycerophospholipid flippase suggests that the two flippases may

operate by different mechanisms to provide a low energy path for lipid translocation across the membrane. Unspecific flipping may involve a mechanism in which the flippase protein would destabilize adjacent phospholipids, allowing them to "slip" into a central transition state, parallel to the plane of the bilayer, from which they could "pop" into either leaflet (32). It may not be possible for the glycerophospholipid flippase to translocate DLOs by such a mechanism because of the charge of the DLO diphosphate bridge, or constraints imposed by the conformation of dolichol. In contrast to this scenario for relatively unspecific flipping, the highly specific translocation of M5-DLO by the DLO flippase suggests binding of lipid, followed by release into the opposing membrane leaflet. Although more work will be necessary before these suggestions can be evaluated, it seems likely that the DLO flippase and the glycerophospholipid flippase operate by distinct mechanisms.

## Materials and Methods

For more detailed descriptions of methods used, see supporting information (SI) *Text*.

**Triton X-100 Solubilized Rat Liver ER Membrane Proteins.** Rough ER was isolated from a rat liver homogenate as described (10) and washed with 0.5 M Tris-HCl, pH 7.2. The washed membranes were treated with ice-cold 0.2% (wt/vol) Triton X-100, 100 mM NaCl, and 10 mM HEPES-NaOH, pH 7.4, to extract soluble luminal contents (33), then incubated with ice-cold 1% (wt/vol) Triton X-100, 100 mM NaCl, and 10 mM HEPES-NaOH, pH 7.4, to solubilize ER membrane proteins. The Triton extract (TE; typically ≈1.5 mg/ml protein) was either used directly for fractionation, or snap-frozen and stored at −80 °C for future use.

**Radiolabeled Dolichol-linked Oligosaccharides.** Radiolabeled dolichol-linked oligosaccharides ([$^3$H]DLO) were isolated from [2-$^3$H]mannose labeled yeast glycosylation mutants as described (15, 34) (Table S1). Nonbiosynthetic [$^3$H]DLO intermediates (iM4- and iM5-DLO) were prepared by treating higher-order [$^3$H]DLOs ([$^3$H]M6-DLO and [$^3$H]M9-DLO, respectively) with *T. reesei* 1,2-α-D-mannosidase (see below). [$^3$H]DLOs were stored in chloroform/methanol/water 10/10/3 (vol/vol/vol) at −20 °C. Radiochemical purity of [$^3$H]DLOs was established by thin layer chromatography (TLC). DLO glycan headgroups were released by mild acid hydrolysis and subjected to methylation linkage analysis according to Ciucanu and Kerek (35). Partially methylated mannose derivatives were resolved by TLC.

**Reconstitution of Proteoliposomes.** Proteoliposomes were reconstituted according to Lévy *et al.* (15, 36). Egg PC (4.1 μmol) in $CHCl_3$ and trace amounts of [$^3$H]DLOs (≈10,000 cpm) in CMW 10/10/3 (vol/vol/v) were dried under nitrogen and solubilized in 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, and 1% (wt/vol) TX-100. The solution was then mixed with either TE or a flippase-enriched fraction as required, in a final volume of 1 ml. Unilamellar vesicles (≈175 nm diameter size) were generated after detergent removal with washed SM-2 Biobeads (≈300 mg) (15, 19, 36). For all assays, protein-free liposomes were reconstituted in parallel. The protein/phospholipid ratio (PPR; mg of protein/mmol of phospholipid) of the vesicles was determined as described. Reconstitution efficiency was similar for all [$^3$H]DLOs.

**Assay for DLO Flipping in Proteoliposomes.** Vesicles reconstituted from egg PC, TE/flippase-enriched fractions and trace amounts of [$^3$H]DLOs were assayed for DLO flip-flop by using a Con A binding assay as previously described (14, 15).

**Chromatography of TE on Cibacron Blue Dye Resin.** TE (~1.5 mg/ml) was adjusted to 10 mM HEPES-NaOH, pH 7.4, 50 mM NaCl, and 1% (wt/vol) TX-100, loaded onto an immobilized reactive dye column (3.0 ml bed volume; pre-equilibrated with 10 column volumes of 10 mM HEPES-NaOH, pH 7.4, 50 mM NaCl, and 1% (wt/vol) TX-100), and rotated end-over-end for 1.5 h at 4 °C after which the resin was washed to generate a flow-through fraction. Bound proteins were eluted sequentially in six steps with 0.1, 0.3, 0.4, 0.5, 0.75 M, and 1.5 M NaCl supplemented with 10 mM HEPES-NaOH, pH 7.4, and 1% (wt/vol) TX-100. The load, flow-through and salt-eluted fractions were adjusted to 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, and 1% (wt/vol) TX-100 by desalting, then reconstituted into proteoliposomes and tested for flippase activity.

**ACKNOWLEDGMENTS.** We thank Christian Frank (Fundación Caubet-CIMERA Illes Balears, Mallorca, Spain) and Markus Aebi (ETH Zurich, Switzerland) for yeast strains, Nico Callewaert (University of Ghent, Belgium) for *T. reesei* 1,2-α-D-mannosidase, Bobby Ng and Hudson Freeze for HPLC analyses of DLO headgroups, Zeynep Turan (Weill Cornell Medical College, New York, NY) for rat liver ER, Jeremy Dittman for helpful discussions, and Arun Radhakrishnan and Jeff Rush for comments on the manuscript. This work was supported by the Cornell-Rockefeller-Sloan-Kettering Tri-Institutional Training Program in Chemical Biology and National Institutes of Health Grant GM71041.

1. Hirschberg CB, Snider MD (1987) Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu Rev Biochem* 56:63–87.
2. Lennarz WJ (1987) Protein glycosylation in the endoplasmic reticulum: Current topological issues. *Biochemistry* 26:7205–7210.
3. Schenk B, Fernandez F, Waechter CJ (2001) The ins(ide) and out(side) of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum. *Glycobiology* 11:61R–70R.
4. Helenius J, Aebi M (2002) Transmembrane movement of dolichol linked carbohydrates during N-glycoprotein biosynthesis in the endoplasmic reticulum. *Semin Cell Dev Biol* 13:171–178.
5. Orlean P, Menon AK (2007) Thematic review series: Lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: How we learned to stop worrying and love glycophospholipids. *J Lipid Res* 48:993–1011.
6. Halter D, *et al.* (2007) Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. *J Cell Biol* 179:101–115.
7. Backer JM, Dawidowicz EA (1987) Reconstitution of a phospholipid flippase from rat liver microsomes. *Nature* 327:341–343.
8. Bishop WR, Bell RM (1985) Assembly of the endoplasmic reticulum phospholipid bilayer: The phosphatidylcholine transporter. *Cell* 42:51–60.
9. Marx U, *et al.* (2000) Rapid flip-flop of phospholipids in endoplasmic reticulum membranes studied by a stopped-flow approach. *Biophys J* 78:2628–2640.
10. Menon AK, Watkins WE, Hrafnsdóttir S (2000) Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmic reticulum. *Curr Biol* 10:241–252.
11. Pomorski T, Menon AK (2006) Lipid flippases and their biological functions. *Cell Mol Life Sci* 63:2908–2921.
12. Kornberg RD, McConnell HM (1971) Inside-outside transitions of phospholipids in vesicle membranes. *Biochemistry* 10:1111–1120.
13. Liu J, Conboy JC (2005) 1,2-diacyl-phosphatidylcholine flip-flop measured directly by sum-frequency vibrational spectroscopy. *Biophys J* 89:2522–2532.
14. Frank CG, Sanyal S, Rush JS, Waechter CJ, Menon AK (2008) Does Rft1 flip an N-glycan lipid precursor? *Nature* 454:E3–4; discussion E4–5.
15. Sanyal S, Frank CG, Menon AK (2008) Distinct flippases translocate glycerophospholipids and oligosaccharide diphosphate dolichols across the endoplasmic reticulum. *Biochemistry* 47:7937–7946.
16. Helenius J, *et al.* (2002) Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein. *Nature* 415:447–450.
17. Vehring S, *et al.* (2007) Flip-flop of fluorescently labeled phospholipids in proteoliposomes reconstituted with Saccharomyces cerevisiae microsomal proteins. *Eukaryot Cell* 6:1625–1634.
18. Gummadi SN, Menon AK (2002) Transbilayer movement of dipalmitoylphosphatidylcholine in proteoliposomes reconstituted from detergent extracts of endoplasmic reticulum. Kinetics of transbilayer transport mediated by a single flippase and identification of protein fractions enriched in flippase activity. *J Biol Chem* 277:25337–25343.
19. Chang QL, Gummadi SN, Menon AK (2004) Chemical modification identifies two populations of glycerophospholipid flippase in rat liver ER. *Biochemistry* 43:10710–10718.
20. Vishwakarma RA, *et al.* (2005) New fluorescent probes reveal that flippase-mediated flip-flop of phosphatidylinositol across the endoplasmic reticulum membrane does not depend on the stereochemistry of the lipid. *Org Biomol Chem* 3:1275–1283.
21. Snider MD, Rogers OC (1984) Transmembrane movement of oligosaccharide-lipids during glycoprotein synthesis. *Cell* 36:753–761.
22. Maras M, *et al.* (2000) Molecular cloning and enzymatic characterization of a Trichoderma reesei 1,2-alpha-D-mannosidase. *J Biotechnol* 77:255–263.
23. Kim S, *et al.* (2000) Dolichol phosphate mannose synthase (DPM1) mutations define congenital disorder of glycosylation Ie (CDG-Ie). *J Clin Invest* 105:191–198.
24. Naismith JH, Field RA (1996) Structural basis of trimannoside recognition by concanavalin A. *J Biol Chem* 271:972–976.
25. Cueva R, Cotano C, Larriba G (1998) N-glycosylation by transfer of GlcNAc2 from dolichol-PP-GlcNAc2 to the protein moiety of the major yeast exoglucanase. *Yeast* 14:773–781.
26. Jackson BJ, Warren CD, Bugge B, Robbins PW (1989) Synthesis of lipid-linked oligosaccharides in *Saccharomyces cerevisiae*: Man2GlcNAc2 and Man1GlcNAc2 are transferred from dolichol to protein in vivo. *Arch Biochem Biophys* 272:203–209.
27. Harford JB, Waechter CJ (1979) Transfer of N, N′-diacetylchitobiose from dolichyl diphosphate into a gray matter membrane glycoprotein. *Arch Biochem Biophys* 197:424–435.
28. Hanover JA, Lennarz WJ (1979) The topological orientation of N,N′-diacetylchitobiosylpyrophosphoryldolichol in artificial and natural membranes. *J Biol Chem* 254:9237–9246.
29. Abeijon C, Hirschberg CB (1990) Topography of initiation of N-glycosylation reactions. *J Biol Chem* 265:14691–14695.

30. Cipollo JF, Trimble RB, Chi JH, Yan Q, Dean N (2001) The yeast ALG11 gene specifies addition of the terminal alpha 1,2-Man to the Man5GlcNAc2-PP-dolichol N-glycosylation intermediate formed on the cytosolic side of the endoplasmic reticulum. *J Biol Chem* 276:21828–21840.
31. Turco SJ, Stetson B, Robbins PW (1977) Comparative rates of transfer of lipid-linked oligosaccharides to endogenous glycoprotein acceptors in vitro. *Proc Natl Acad Sci USA* 74:4411–4414.
32. Kol MA, de Kroon AI, Killian JA, de Kruijff B (2004) Transbilayer movement of phospholipids in biogenic membranes. *Biochemistry* 43:2673–2681.
33. Kreibich G, Sabatini DD (1974) Procedure for the selective release of content from microsomal vesicles without membrane disassembly. *Methods Enzymol* 31:215–225.
34. Zufferey R, *et al.* (1995) STT3, a highly conserved protein required for yeast oligosaccharyl transferase activity in vivo. *EMBO J* 14:4949–4960.
35. Ciucanu I, Kerek F (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* 131:209–217.
36. Levy D, Bluzat A, Seigneuret M, Rigaud JL (1990) A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal. *Biochim Biophys Acta* 1025:179–190.

# Supporting Information

Sanyal and Menon 10.1073/pnas.0810225106

## SI Text

**Materials.** Egg phosphatidylcholine (ePC) was from Avanti Polar Lipids. ULTROL grade Triton X-100 (TX-100) was from Roche Molecular Biochemicals. The micro BCA protein assay kit and BSA protein standard were from Pierce. D-[2-$^3$H]mannose (≈20 Ci/mmol) was from American Radiolabeled Chemicals. Frozen rat-livers were from Pel Freez. *Trichoderma reesei* 1,2-α-D-mannosidase (1) was a gift from Prof. Nico Callewaert (University of Ghent, Belgium). Concanavalin A (Con A), Fast-flow Cibacron blue 3GA resin and routine chemicals were from Sigma. Standard yeast media and growth conditions were used.

**Isolation of Rat Liver ER and Preparation of the Triton X-100 Extract (TE).** Rough ER was isolated from a rat liver homogenate as described (2), and suspended at approximately 10 mg/ml. The membranes were incubated with an equal volume of 1 M Tris-HCl, pH 7.2, for 30 min on ice, then recovered by centrifugation (Beckman Ti50.2 rotor, 35,000 rpm, 1 h, 4 °C) through a 0.5 M sucrose cushion as described (2). The Tris-washed membranes (TWER) were suspended at approximately 10 mg/ml in 0.25 M sucrose and 10 mM Hepes-NaOH, pH 7.4, snap-frozen in liquid nitrogen and stored at −80 °C. Approximately 8 mg TWER were recovered per 5 g liver.

To prepare TE, TWER membranes were thawed rapidly and combined with an equal volume of ice-cold 0.2% (wt/vol) Triton X-100, 100 mM NaCl, and 10 mM Hepes-NaOH, pH 7.4, to extract soluble luminal contents (3). The sample was incubated for 30 min at 4 °C before being centrifuged (Beckman TLA 100.3 rotor, 70,000 rpm, 30 min, 4 °C). The supernatant was discarded. The pellet was resuspended in 100 mM NaCl, and 10 mM HEPES-NaOH, pH 7.4, to the same volume as the original membranes, then mixed with an equal volume of ice-cold 2% (wt/vol) Triton X-100, 100 mM NaCl, and 10 mM HEPES-NaOH, pH 7.4. The mixture was left on ice for 30 min with occasional mixing before being centrifuged in a Beckman TLA 100.3 rotor at 70,000 rpm for 30 min at 4 °C. The resulting supernatant (TE; typically ≈1.5 mg/ml protein) was either used directly for fractionation and/or reconstitution into proteoliposomes, or snap-frozen and stored at −80 °C for future use. Approximately 30% of TWER protein was recovered in the TE.

**Radiolabeled Dolichol-Linked Oligosaccharides.** Radiolabeled dolichol-linked oligosaccharides ([$^3$H]DLO) were isolated from [2-$^3$H]mannose labeled yeast strains as described (4, 5). Strains are listed in Table S1. Strains YG248 and YG1057 were used to prepare [$^3$H]M5-DLO. [$^3$H]M6-DLO was prepared from YG414, [$^3$H]M7-DLO from YG1056, [$^3$H]M9-DLO from YG592, and [$^3$H]G2M9-DLO from YG428. Strain YG1146 was used to isolate [$^3$H]M3-DLO and a nonbiosynthetic [$^3$H]M7-DLO isomer (iM7-DLO). Other nonbiosynthetic [$^3$H]DLO intermediates (iM4- and iM5-DLO) were prepared by treating higher-order [$^3$H]DLOs with *T. reesei* 1,2-α-D-mannosidase as described below. Extracted [$^3$H]DLOs were stored in chloroform/methanol/water 10/10/3 (vol/vol/vol) at −20 °C. Radiochemical purity of [$^3$H]DLOs was established by thin layer chromatography (TLC) on Silica-60 plates (Merck) developed in chloroform/methanol/water (CMW) 10/10/3 (vol/vol/vol); radioactivity on the TLC-plates was detected by using a TLC scanner, or by exposing the plate to a tritium screen for visualization with a phosphorimager.

DLO glycan head groups were released by mild acid hydrolysis in 0.1 N HCl and subjected to methylation linkage analysis according to the procedure of Ciucanu and Kerek (6). Partially methylated mannose derivatives were resolved by TLC on silica-gel 60 plates by using benzene/acetone/water/30% $NH_3$ (50/200/3/1.5, by volume) as the solvent system. Quantitation of the radioactivity recovered in individual partially methylated mannose derivatives indicated that the DLOs were labeled approximately to steady state, allowing their specific radioactivity to be determined.

**Reconstitution of Proteoliposomes.** Proteoliposomes were reconstituted according to Lévy *et al.* (7). Egg PC (4.1 μmol) in $CHCl_3$ and trace amounts of [$^3$H]DLOs (≈10,000 cpm) in CMW 10/10/3 (vol/vol/vol) were dried under nitrogen and solubilized in 10 mM Hepes-NaOH, pH 7.4, 100 mM NaCl, and 1% (wt/vol) TX-100. The solution was then mixed with either TE or a flippase-enriched fraction as required in a final volume of 1 ml. Unilamellar vesicles (≈175 nm diameter size) were generated after detergent removal with washed SM-2 Biobeads (≈300 mg) as described previously (5, 7, 8). For all assays, protein-free liposomes were reconstituted in parallel by omitting TE. The protein/phospholipid ratio (PPR; mg of protein/mmol of phospholipid) of the vesicles was determined by using Kaplan and Pedersen's method to measure protein concentrations and a phosphate assay to quantitate total phospholipids (9, 10). Reconstitution efficiency was similar for all [$^3$H]DLOs.

**Assay for DLO Flipping in Proteoliposomes.** Vesicles reconstituted from egg PC, TE/flippase-enriched fractions and trace amounts of [$^3$H]DLOs were assayed for DLO flip-flop by using a Con A-binding assay as previously described (5, 11). To determine the kinetics of DLO flipping, data were corrected by first subtracting the amount of DLO captured in the absence of flipping and then normalizing to the total extent of flipping. The data were analyzed by using a simple first order kinetic model, assuming that Con A is in considerable excess over the amount of DLO present in the assay.

**Chromatography of TE on Cibacron Blue Dye Resin.** TE (≈1.5 mg/ml) was adjusted to 10 mM Hepes-NaOH, pH 7.4, 50 mM NaCl, and 1% (wt/vol) TX-100. The sample (20 ml) was loaded onto an immobilized reactive dye column [3.0-ml bed volume; preequilibrated with 10 column volumes of 10 mM HEPES-NaOH, pH 7.4, 50 mM NaCl, and 1% (wt/vol) TX-100] and rotated end-over-end for 1.5 h at 4 °C after which the resin was washed with the equilibration buffer to generate a flow-through fraction. Bound proteins were eluted sequentially in six steps with 0.1, 0.3, 0.4, 0.5, 0.75 M, and 1.5 M NaCl supplemented with 10 mM HEPES-NaOH, pH 7.4, and 1% (wt/vol) TX-100. The load, flow-through, and salt-eluted fractions were desalted over Biogel P6 spin columns to readjust to 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, and 1% (wt/vol) TX-100, then reconstituted into proteoliposomes, and tested for flippase activity.

**Digestion of [$^3$H]DLOs with *Trichoderma reesei* 1,2-α-D-Mannosidase (Trαm).** iM4-DLO and iM5-DLO were generated by treating [$^3$H]M6-DLO and [$^3$H]M9-DLO, respectively, with *T. reesei* 1,2-α-D-mannosidase. [$^3$H]DLOs (≈100,000 cpm) were dried under a stream of nitrogen and resuspended in 200 μl of 20 mM HOAc/NaOAc, 10 mM $CaCl_2$, and 0.1% (wt/vol) TX-100, pH 5.0. Trαm (44 mU) was added, and the reaction mixture was incubated at 37 °C. After 24 h, a second aliquot of the enzyme was added, and the reaction was allowed to proceed for a further

24 h before being terminated by adding 25 μl of 0.3 M NaOH. Lipids were extracted by partitioning between 1-butanol and water. The butanol extracts were pooled, back-extracted with 1-butanol-saturated water, dried and dissolved in 500 μl of chloroform/methanol/water 10/10/3 (vol/vol/vol). Radiochemical purity was determined by analyzing 5 μl of the sample by TLC (Silica gel-60; solvent: CMW 10/10/3 (vol/vol/vol)). Where multiple peaks were observed, the sample was run on a preparative scale and the major peak was scraped and eluted from the silica gel by using CMW 10/10/3 and reanalyzed by using TLC.


1. Maras M, *et al.* (2000) Molecular cloning and enzymatic characterization of a Trichoderma reesei 1,2-alpha-D-mannosidase. *J Biotechnol* 77:255–263.
2. Menon AK, Watkins WE, Hrafnsdóttir S (2000) Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmic reticulum. *Curr Biol* 10:241–252.
3. Kreibich G, Sabatini DD (1974) Procedure for the selective release of content from microsomal vesicles without membrane disassembly. *Methods Enzymol* 31:215–225.
4. Zufferey R, *et al.* (1995) STT3, a highly conserved protein required for yeast oligosaccharyl transferase activity in vivo. *EMBO J* 14:4949–4960.
5. Sanyal S, Frank CG, Menon AK (2008) Distinct flippases translocate glycerophospholipids and oligosaccharide diphosphate dolichols across the endoplasmic reticulum. *Biochemistry* 47:7937–7946.
6. Ciucanu I, Kerek F (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* 131:209–217.
7. Levy D, Bluzat A, Seigneuret M, Rigaud JL (1990) A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal. *Biochim Biophys Acta* 1025:179–190.
8. Chang QL, Gummadi SN, Menon AK (2004) Chemical modification identifies two populations of glycerophospholipid flippase in rat liver ER. *Biochemistry* 43:10710–10718.
9. Kaplan RS, Pedersen PL (1989) Sensitive protein assay in presence of high levels of lipid. *Methods Enzymol* 172:393–399.
10. Rouser G, Fleischer S, Yamamoto A (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5:494–496.
11. Frank CG, Sanyal S, Rush JS, Waechter CJ, Menon AK (2008) Does Rft1 flip an N-glycan lipid precursor? *Nature* 454: E3–4; discussion E4–5.
12. Vijayraghavan U, Company M, Abelson J (1989) Isolation and characterization of pre-mRNA splicing mutants of Saccharomyces cerevisiae. *Gene Dev* 3:1206–1216.
13. Aebi M, Gassenhuber J, Domdey H, te Heesen S (1996) Cloning and characterization of the ALG3 gene of Saccharomyces cerevisiae. *Glycobiology* 6:439–444.
14. Burda P, *et al.* (1996) Stepwise assembly of the lipid-linked oligosaccharide in the endoplasmic reticulum of Saccharomyces cerevisiae: Identification of the ALG9 gene encoding a putative mannosyl transferase. *Proc Natl Acad Sci USA* 93:7160–7165.
15. Burda P, Aebi M (1998) The ALG10 locus of Saccharomyces cerevisiae encodes the alpha-1,2 glucosyltransferase of the endoplasmic reticulum: The terminal glucose of the lipid-linked oligosaccharide is required for efficient N-linked glycosylation. *Glycobiology* 8:455–462.
16. Jakob CA, Burda P, te Heesen S, Aebi M, Roth J (1998) Genetic tailoring of N-linked oligosaccharides: The role of glucose residues in glycoprotein processing of Saccharomyces cerevisiae in vivo. *Glycobiology* 8:155–164.
17. Helenius J, *et al.* (2002) Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein. *Nature* 415:447–450.

**Table S1. Yeast strains used in this study**

| Strain | Genotype | Source |
|---|---|---|
| SS328 | *MATα ade2–101 his3Δ200 ura3–52 lys2–801* | 12 |
| YG248 | *MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δalg3::HIS3* | 13 |
| YG1056 | *MATα ade2–101 ura3–52 his3Δ200 Δalg12::kanMX4 Δalg5::HIS3* | Aebi laboratory strain collection |
| YG1057 | *MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δalg3::HIS3 Δalg5::HIS3* | Aebi laboratory strain collection |
| YG414 | *MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg9::KanMX* | 14 |
| YG428 | *MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg10::KanMX* | 15 |
| YG592 | *MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δalg6::HIS3* | 16 |
| YG1146 | *MATα ade2–101 ura3–52 his3Δ200 lys2–801 GalProRFT1:KanMX Δalg11::HIS3* | 17 |

All strains used are in the SS328/SS330 strain background