Dolichol-linked oligosaccharide selection by the oligosaccharyltransferase in protist and fungal organisms

Daniel J. Kelleher,1 Sulagna Banerjee,2 Anthony J. Cura,1 John Samuelson,2 and Reid Gilmore1

1Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605
2Department of Molecular and Cell Biology, Goldman School of Dental Medicine, Boston University, Boston, MA 02118

The dolichol-linked oligosaccharide Glc3Man9GlcNAc2-PP-Dol is the in vivo donor substrate synthesized by most eukaryotes for asparagine-linked glycosylation. However, many protist organisms assemble dolichol-linked oligosaccharides that lack glucose residues. We have compared donor substrate utilization by the oligosaccharyltransferase (OST) from Trypanosoma cruzi, Entamoeba histolytica, Trichomonas vaginalis, Cryptococcus neoformans, and Saccharomyces cerevisiae using structurally homogeneous dolichol-linked oligosaccharides as well as a heterogeneous dolichol-linked oligosaccharide library. Our results demonstrate that the OST from diverse organisms utilizes the in vivo oligosaccharide donor in preference to certain larger and/or smaller oligosaccharide donors. Steady-state enzyme kinetic experiments reveal that the binding affinity of the tripeptide acceptor for the protist OST complex is influenced by the structure of the oligosaccharide donor. This rudimentary donor substrate selection mechanism has been refined in fungi and vertebrate organisms by the addition of a second, regulatory dolichol-linked oligosaccharide binding site, the presence of which correlates with acquisition of the SWP1/ribophorin II subunit of the OST complex.

Introduction

The eukaryotic oligosaccharyltransferase (OST) transfers pre-assembled oligosaccharides onto asparagine residues as nascent polypeptides are translocated across the rough ER membrane (for review see Kelleher and Gilmore, 2006). The consensus site for N-linked glycosylation in eukaryotic organisms is conserved and corresponds to the simple tripeptide sequence N-X-T/S, where X can be any residue except proline (Gavel and Von Heijne, 1990).

The dolichol-linked oligosaccharide donor assembled by most eukaryotes for N-linked glycosylation is the dolichol pyrophosphate–linked oligosaccharide Glc3Man9GlcNAc2-PP-Dol (abbreviated here as Glc3Man9GlcNAc2-PP-Dol). Synthesis of the dolichol-linked oligosaccharide (OS-PP-Dol) donor occurs by the stepwise addition of monosaccharide residues onto the dolichol-pyrophosphate carrier by a family of ER-localized membrane bound glycosyltransferases (asparagine-linked glycosylation [ALG] gene products; for review see Burda and Aebl, 1999). Man,GlcNAc2-PP-Dol (M5GN2-PP-Dol) is assembled on the cytoplasmic face of the ER membrane, with UDP-GlcNAc and GDP-Man serving as monosaccharide donors. Man-P-Dol and Glc-P-Dol are the donors for the luminally oriented glycosyltransferases that add four mannose and three glucose residues to OS-PP-Dol assembly intermediates within the ER lumen. Depletion of the yeast Rft1 protein causes severe hypoglycosylation of proteins and accumulation of Man5GlcNAc2-PP-Dol (Helenius et al., 2002) even though Alg3p, not Rft1p, is the mannosyltransferase that adds the sixth mannose residue. Rft1p has been proposed to flip cytosolically oriented M5GN2-PP-Dol across the ER membrane (Helenius et al., 2002).

Certain kinetoplastids (Trypanosoma cruzi and Leishmania mexicana) and the ciliate Tetrahymena pyriformis assemble OS-PP-Dol compounds that lack the glucose residues (M5GN2-PP-Dol by T. cruzi) and/or the mannose residues (G5M2GN2-PP-Dol by T. pyriformis and M5GN2-PP-Dol by L. mexicana) that are transferred by the luminally oriented ALG gene products (de la Canal and Parodi, 1987; Parodi, 1993). Searches of fully sequenced genomes using yeast ALG proteins as query sequences has revealed considerable diversity in OS-PP-Dol selection by the oligosaccharyltransferase in protist and fungal organisms.
biosynthesis amongst unicellular organisms (Samuelson et al., 2005). Biochemical studies have confirmed bioinformatic predictions that *Giardia lamblia* synthesizes GN2-PP-Dol, *Trichomonas vaginalis* and *Entamoeba histolytica* synthesize M5GN2-PP-Dol, and the pathogenic fungi *Candida neoformans* synthesizes M6GN2-PP-Dol (Fig. 1; Samuelson et al., 2005). The diversity of eukaryotic OS-PP-Dol donors was proposed to have occurred by secondary loss of ALG genes during the evolution of current eukaryotes from a last common ancestor with a complete ALG pathway (Samuelson et al., 2005).

In fungi and vertebrate organisms, the OST is an oligomer composed of seven to eight nonidentical subunits (for review see Kelleher and Gilmore, 2006). Of the eight *Saccharomyces cerevisiae* OST subunits (Stt3p, Ost1p, Ost2p, Ost3p or Ost6p, Ost4p, Ost5p, Wbp1p, and Swp1p), five are encoded by essential yeast genes (STT3, OST1, OST2, WBP1, and SWP1). With the exception of STT3, which contains the enzyme active site (Yan and Lennarz, 2002; Kelleher et al., 2003; Nilsson et al., 2003), relatively little is known about the roles of the essential or non-essential subunits (for review see Kelleher and Gilmore, 2006). Vertebrate, plant, and insect genomes encode two forms of the catalytic subunit that are designated as STT3A and -B (Kelleher et al., 2003; Koika et al., 2003). The canine STT3 homologues are assembled with a shared set of noncatalytic subunits (ribophorin I [Ost1 homologue], ribophorin II [Swp1], OST48 [Wbp1], DAD1 [Ost2], and TUSC3 or IAP [Ost3 or -6] and OST4) to generate OST isoforms with kinetically distinct properties (Kelleher et al., 2003). Protein and DNA sequence database searches of fully sequenced eukaryotic genomes using the yeast and human OST subunits as query sequences suggest that the OST in protist organisms has a simpler subunit composition (Fig. 1; Kelleher and Gilmore, 2006). The genomes of *G. lamblia* and the kinetoplastists *T. cruzi* and *Trypanosoma brucei* encode several different STT3 proteins (Samuelson et al., 2005), yet lack genes encoding the noncatalytic subunits. Four-subunit complexes, consisting of STT3, OST1, OST2, and WBP1, are predicted for *T. vaginalis* and *E. histolytica*. A six-subunit complex (STT3, OST1, OST2, OST3, OST4, and WBP1) is predicted for *Cryptosporidium parvum*. The *C. neoformans* genome encodes readily identifiable homologues of all *S. cerevisiae* OST subunits with the exception of Ost5p (Fig. 1).

The absence of glucose residues on OS-PP-Dol compounds assembled by most protists and *C. neoformans* is of particular interest because the terminal glucose residue on G1-3M9GN2-PP-Dol is a critical substrate recognition determinant for the OST. OS-PP-Dol assembly intermediates that lack the terminal glucose residue are transferred less rapidly by the vertebrate and yeast OST (Turco et al., 1977; Trimble et al., 1980; Bosch et al., 1988; Karaoglu et al., 2001; Kelleher et al., 2003), thereby minimizing synthesis of glycoproteins with aberrant oligosaccharide structures. Glycosylation of proteins with an oligosaccharide assembly intermediate could interfere with glycoprotein quality-control pathways in the ER as well as subsequent oligosaccharide-processing reactions in the Golgi complex (for review see Helenius and Aebi, 2004). Cellular defects in G1M6GN2-PP-Dol biosynthesis cause a family of inherited diseases (congenital disorders of glycosylation [CDG-II]) due to hypoglycosylation of nascent glycoproteins by the OST in cells that accumulate an assembly intermediate or are unable to maintain a normal concentration of fully assembled G1M6GN2-PP-Dol (for review see Freeze and Aebi, 2005).

Preferential utilization of G1M6GN2-PP-Dol by the yeast and vertebrate OST occurs by allosteric interactions between a regulatory OS-PP-Dol binding site and the active site subunit, as well as by oligosaccharide structure-mediated alterations in tripeptide acceptor binding affinity (Karaoglu et al., 2001; Kelleher et al., 2003). Kinetic analysis of the purified canine OST isoforms has suggested that the regulatory OS-PP-Dol binding site is not located on STT3A or -B, but is instead associated with one or more of the noncatalytic subunits (Kelleher et al., 2003).

Does the OST from organisms that synthesize nonglucosylated OS-PP-Dols transfer the in vivo donor in preference to OS-PP-Dol assembly intermediates or G1M6GN2-PP-Dol? Previous studies indicate that the *T. cruzi* OST transfers glucosylated (G1M6GN2-PP-Dol) and large nonglucosylated (M5GN2-PP-Dol) donors at similar rates in vitro (Bosch et al., 1988), suggesting that the *T. cruzi* OST is nonselective. Can biochemical analysis of the OST from primitive eukaryotes reveal properties of the higher eukaryotic OST that arose as additional subunits were added to the STT3 catalytic core? Here, we report a comparison of the OST from *T. vaginalis*, *E. histolytica*, *T. cruzi*, *C. neoformans*, and *S. cerevisiae*, with emphasis placed upon an analysis of donor

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Figure 1. OS-PP-Dol donors and predicted subunit compositions of the OST from selected eukaryotes. The left portion of A–D shows the oligosaccharide structure of the in vivo donor for N-linked glycosylation. N-acetylglucosamine residues are designated by squares, mannose residues are shown as circles, and glucose residues are shown as triangles. Red saccharides are transferred by cytoplasmically oriented ALG gene products, and blue residues are transferred by luminally oriented ALG gene products. The right section of each panel shows the predicted (A, *T. cruzi*; B, *C. neoformans*; or C, *T. vaginalis* and *E. histolytica*) or experimentally determined (D, *S. cerevisiae*) composition of the OST complex. The color code of the subunits (red, green, and blue) designates subcomplexes detected in higher eukaryotes (Karaoglu et al., 1997; Spirig et al., 1997). The yellow bar designates the ER membrane.
substrate selection. Our results support the hypothesis that terminal mannose residues on the OS-PP-Dol are important for donor substrate recognition by the OST in organisms that assemble non-glucosylated OS-PP-Dol compounds. Cooperative OS-PP-Dol binding, a feature of the yeast and canine OST complex that facilitates exquisite G3M9GN2-PP-Dol selection, is not a property of the predicted one- and four-subunit protist OST complexes.

Results

Donor substrate selection by the OST

Is preferential utilization of the in vivo oligosaccharide donor an OST property that is restricted to eukaryotes that assemble triglucosylated OS-PP-Dols? To address this question, the OST from selected protists and C. neoformans was assayed using a synthetic tripeptide acceptor and a heterogeneous bovine OS-PP-Dol library that consists of donors that range in size between M1GN2-PP-Dol and G3M9GN2-PP-Dol. Enzyme concentrations were adjusted to ensure that a maximum of 3% of the total donor substrate was converted into glycopeptides. Radiolabeled glycopeptide products that were captured with an immobilized lectin (ConA Sepharose) were subsequently eluted and resolved by high-pressure liquid chromatography (HPLC) according to the number of saccharide residues (Fig. 2). As expected, G3M9GN2-PP-Dol was the most abundant product when the purified S. cerevisiae OST was assayed (Fig. 2 A). In contrast, G3M9GN2-NTY was less abundant in the T. cruzi glycopeptide products (Fig. 2 B) and barely detectable in glycopeptide products derived from assays of the T. vaginalis (Fig. 2 C), C. neoformans (Fig. 2 D), or E. histolytica (not depicted) OST. The composition of the OS-PP-Dol donor library was determined as described previously (Kelleher et al., 2001) by incubating an excess of the purified yeast OST with a low quantity of the donor substrate (OST endpoint assay; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200611079/DC1). A normalized initial transfer rate (glycosylated tripeptide [OS-NYT]/OS-PP-Dol; Fig. 2, E and F) was calculated for the eight most abundant donors by dividing the glycopeptide product composition by the composition of the OS-PP-Dol donor substrate library. A normalized initial transfer rate of 1 (Fig. 2, E and F, dashed lines) indicates nonselective utilization of a donor substrate relative to the total donor pool.

The T. vaginalis and E. histolytica OST transfer the mannosylated donors (M1M0GN2-PP-Dol) threefold more rapidly than G3M9GN2-PP-Dol (Fig. 2, E and F). Although the T. cruzi OST utilizes compounds ranging in size between M1GN2-PP-Dol and G3M9GN2-PP-Dol at rates similar to those reported previously (Bosch et al., 1988), OS-PP-Dol donors with fewer mannose residues (M1M0GN2-PP-Dol) were transferred less rapidly (Fig. 2 F). The C. neoformans OST showed preferential utilization of the in vivo donor (M1GN2-PP-Dol) relative to assembly intermediates (M1M0GN2-PP-Dol) and the glucosylated donor (Fig. 2 F).

Structural determinants of donor substrate selection

Careful inspection of the glycopeptide elution profiles (Fig. 2, A–D) revealed that several of the larger glycopeptide peaks (e.g., M1G2-NTY) have prominent shoulders, suggesting oligosaccharide structural heterogeneity. The structural diversity of the OS-PP-Dol donor substrate library is thought to arise by exposure of G3M9GN2-PP-Dol to cellular glucosidases and mannosidases during isolation (Kelleher et al., 2001). Mannosidase degradation of M1G2-PP-Dol (Fig. 3 A, compound a) could yield seven M1G2-PP-Dol isomers (Fig. 3 A, compounds c–i) that differ from biosynthetic M1G2-PP-Dol (Fig. 3 A, compound b). Biosynthetic M1G2-PP-Dol (Fig. 3 A, compound b) can be readily distinguished from these other isomers by digestion with α-1,2 mannosidase, as it is the only isomer that has two α-1,2–linked mannose residues (Fig. 3 A, red circles). M1G2, glycopeptides produced in an OST endpoint assay were purified by preparative HPLC (Fig. 3 B, left) and digested to completion with α-1,2 mannosidase. Resolution of
Figure 3. Reduced transfer of biosynthetic M5GN2-PP-Dol by the T. cruzi OST. (A) Biosynthetic M5GN2-PP-Dol (b) and M5GN2-PP-Dol isomers (c–i) produced by mannosidase digestion of M5GN2-PP-Dol (a). GlcNAc residues are indicated by squares, α-1,2-linked mannose residues are indicated by red circles, and α-1,3- and α-1,6-linked mannose residues are indicated by open circles. (B) Glycopeptide products obtained in an OST endpoint assay (>95% conversion of OS-PP-Dol to OS-NYT) were resolved by preparative HPLC to isolate the M5GN2-NYT glycopeptide (left). HPLC resolution of the α-1,2 mannosidase digestion products derived from M5GN2-NYT (right). The M5GN2-NYT (M3) peak is derived from isomer b, the M5GN2-NYT (M4) peak is derived from isomers c–h, and the M5GN2-NYT (M5) peak corresponds to isomer i. (C) HPLC profiles of α-1,2 mannosidase digestion products derived from M5GN2-NYT synthesized by the E. histolytica and T. cruzi OST. Redigestion of the M4 peak with α-1,2 mannosidase did not yield smaller products (not depicted); hence, the initial digestion had gone to completion. (D) The distribution of the three isomer classes (2, 1, or 0 α-1,2-linked mannose residues) was calculated for the total M5GN2-PP-Dol pool (OS) and for M5GN2-NYT synthesized by the S. cerevisiae (Sc), T. cruzi (Tc), E. histolytica (Eh), and T. vaginalis (Tv) OST. Values for the OS, Sc, and Tc are means of two independent experiments; error bars designate one of two independent data points. The OS values are derived from two replicates of B.

The digestion products by HPLC (Fig. 3, B) yielded three peaks (M3, M4, and M5) that are derived from M5GN2-PP-NYT isomers that contain 2, 1, or 0 α-1,2-linked mannose residues. Quantification (Fig. 3 C, D, black bars) of two independent experiments revealed that 22% of the M5GN2 glycopeptides were derived from biosynthetic M5GN2-PP-Dol (Fig. 3 A, compound b), 63% from compounds c–h, and 15% from compound i.

If the OST from T. vaginalis, E. histolytica, T. cruzi, or S. cerevisiae selects biosynthetic M5GN2-PP-Dol (Fig. 3 A, compound b) in preference to other M5GN2-PP-Dol isomers, the M5GN2 glycopeptides synthesized in the presence of excess donor substrate should be enriched in glycopeptides that contain two α-1,2-linked mannose residues. To ensure that our glycopeptide product analysis provided a reliable measure of the relative initial transfer rate, the OST assays were terminated when <10% of the total M5GN2-PP-Dol was converted into glycopeptides. Typical HPLC profiles of the α-1,2 mannosidase digestion products of the M5GN2 glycopeptides are shown in Fig. 3 C, and the results from assays of all four organisms are quantified in Fig. 3 D. We observed a very similar distribution of M5GN2 isomers for the donor substrate pool and the initial S. cerevisiae glycopeptide products (Fig. 3 D, compare black and white bars), thereby indicating that the S. cerevisiae OST does not discriminate between M5GN2-PP-Dol isomers. The M5GN2 glycopeptides synthesized by the T. vaginalis and E. histolytica OST also resembled the M5GN2-PP-Dol donor pool; hence, the OST from these organisms does not select biosynthetic M5GN2-PP-Dol in preference to other M5GN2-PP-Dol isomers (Fig. 3 D). M5GN2 glycopeptides synthesized by the T. cruzi OST were twofold deficient in biosynthetic M5GN2-NYT and enriched in one or more M5GN2-NYT isomers that have one α-1,2-linked mannose residue (Fig. 3 D). This result, taken together with a reduced transfer rate for M3-6GN2-PP-Dol relative to M7-9GN2-PP-Dol (Fig. 2 F) by the T. cruzi OST suggests that a terminal α-1,2-linked mannose residue on the B or C antennae of M5GN2-PP-Dol serves as a positive determinant for substrate selection by the T. cruzi OST.

Donor substrate competition experiments were conducted using purified biosynthetic M5GN2-PP-Dol (Fig. 3 A, isomer b), M5GN2-PP-Dol (Fig. 3 A, compound a), and G3M9GN2-PP-Dol. The T. vaginalis OST will synthesize G3M9GN2-NYT when G3M9GN2-PP-Dol is the sole donor substrate (Fig. 4 A, profile a). The absence of the M5GN2-NYT product indicates that the endogenous donor substrate is not abundant in the assay mix relative to the exogenous donor substrate. Analogous results were obtained using detergent extracts prepared from T. cruzi, E. histolytica, and C. neoformans (unpublished data). When the M5GN2-PP-Dol/G3M9GN2-PP-Dol ratio is 2.5:1, the yeast OST primarily synthesized G3M9GN2-NYT, unlike the T. vaginalis OST that synthesized M5GN2-NYT (Fig. 4 A, profiles b and c). Quantification of this competition experiment, as well as additional assays containing 1.5 μM M5GN2-PP-Dol and variable concentrations of G3M9GN2-PP-Dol, showed that donor substrate selection by the T. vaginalis (Fig. 4 B, squares) and S. cerevisiae (Fig. 4 B, circles) OST occurs across a wide range of donor substrate ratios (Fig. 4 B). Additional donor substrate competition experiments were conducted using 1:1 mixtures of the three purified oligosaccharide donors (Fig. 4, C–E). The S. cerevisiae OST selects G3M9GN2-PP-Dol in preference to both nonglucosylated donors (Fig. 4, C and D) but does not discriminate between M5GN2-PP-Dol and M5GN2-PP-Dol (Fig. 4 E). The OST from E. histolytica and T. vaginalis selects both nonglucosylated...
Oligosaccharide donor competition assays. OST activity was assayed using a constant concentration of the acceptor tripeptide (5 μM in A, B, and F and 10 μM in C–E). [A] Glycopeptide products from assays of the T. vaginalis (a and c) or S. cerevisiae (b) OST using 1 μM G3M9GN2-PP-Dol (a) or 0.6 μM G2M9GN2-PP-Dol plus 1.5 μM M5GN2-PP-Dol (b and c) were resolved by HPLC. For clarity, column profiles have been offset on the vertical axis. (B) The T. vaginalis (squares) or S. cerevisiae (circles) OST were assayed using 1.5 μM M5GN2-PP-Dol and increasing concentrations of G3M9GN2-PP-Dol. Glycopeptide products were resolved by HPLC to determine the percentage of M5GN2-NYT. The dashed line indicates the composition [in percentage of M5GN2-PP-Dol] of the donor substrate mixtures. (C–E) Purified S. cerevisiae (Sc) or detergent extracts of C. neoformans (Cn), E. histolytica (Eh), T. vaginalis (Tv), or T. cruzi (Tc) membranes were assayed using the following donor substrate mixtures: 1 μM G3M9GN2-PP-Dol + 1 μM M5GN2-PP-Dol + 1 μM G3M9GN2-PP-Dol, and 1 μM M5GN2-PP-Dol + 1 μM M3GN2-PP-Dol. Glycopeptides were resolved by HPLC to determine product composition. [F] The T. vaginalis (squares) or S. cerevisiae (circles) OST were assayed using the following mixture: (G2M9GN2-PP-Dol/G1M9GN2-PP-Dol/M5GN2-PP-Dol) (G2M9GN2-PP-Dol, G1M9GN2-PP-Dol, and M5GN2-PP-Dol). Glycopeptides were resolved by HPLC (Fig. 4 F, top). The initial transfer rates of ~1 for the S. cerevisiae OST serves as an important control for the observed lower transfer rates of G2M9GN2-PP-Dol and G3M9GN2-PP-Dol relative to M5GN2-PP-Dol by the T. vaginalis OST. Each additional glucose residue on the A branch of the oligosaccharide reduces the normalized initial transfer rate by the T. vaginalis OST.

Kinetic parameters for the OS-PP-Dol donor

Enzyme kinetic experiments suggest that selection of the fully assembled OS-PP-Dol by the yeast or mammalian OST occurs by allosteric communication between a regulatory OS-PP-Dol binding site and the donor substrate binding site on STT3, in addition to oligosaccharide structure–dependent alterations in tripeptide substrate binding affinity (Karaoglu et al., 2001; Kelleher et al., 2003). Nonlinear Lineweaver-Burk plots for the OS-PP-Dol substrate are diagnostic of the cooperative OS-PP-Dol binding kinetics of the yeast and mammalian OST (Karaoglu et al., 2001). Donor substrate saturation experiments for the T. vaginalis (Fig. 5 A), E. histolytica (Fig. 5 C), and T. cruzi enzymes (Fig. 5 D) were conducted using a constant concentration of tripeptide acceptor and increasing concentrations of purified OS-PP-Dols. The linear Lineweaver-Burk plots yield K_m values in the submicromolar range for the in vivo donor substrate. The experimental data for the T. vaginalis OST was replotted as an Eadie-Hofstee plot (Fig. 5 B). The linear Eadie-Hofstee plot for the T. vaginalis OST is inconsistent with cooperative OS-PP-Dol binding kinetics. In contrast, the S. cerevisiae OST binds the same donor substrate (M5GN2-PP-Dol) in a cooperative manner, as revealed by a nonlinear Eadie-Hofstee plot (Fig. 5 B, inset). Additional donor substrate saturation experiments using the nonoptimal donors (M5GN2-PP-Dol for T. cruzi OST and G3M9GN2-PP-Dol for T. vaginalis OST) did not reveal differences in the apparent K_m that could account for the lower transfer rates of the nonoptimal donor substrate (unpublished data). Donor substrate selection by the protist OST does not involve a regulatory OS-PP-Dol binding site, nor is it explained by a reduced affinity for the nonoptimal oligosaccharide donor.

Kinetic parameters for the tripeptide substrate acceptor

Reduced transfer rates for nonoptimal donors by the yeast and mammalian OST is in part explained by a reduced binding affinity for the tripeptide acceptor in the presence of an OS-PP-Dol assembly intermediate (Breuer and Bause, 1995; Gibbs and Coward, 1999; Karaoglu et al., 2001; Kelleher et al., 2003). The T. vaginalis (Fig. 6 A) and T. cruzi (Fig. 6 B) OST were assayed in the presence of a constant concentration of the optimal and nonoptimal oligosaccharide donors and increasing concentrations.
of the tripeptide acceptor. The linear Lineweaver-Burk plots for the tripeptide acceptors were indicative of a single acceptor tripeptide binding site, as observed for the yeast and mammalian OST (Karagolu et al., 2001; Kelleher et al., 2003). The nonoptimal donor substrate (G3M9GN2-PP-Dol for T. vaginalis and M5GN2-PP-Dol for T. cruzi) reduces the binding affinity of the OST for the tripeptide acceptor. In both cases, the threefold decrease in acceptor tripeptide binding affinity is responsible for the reduction in the normalized transfer rate when the acceptor tripeptide is not saturating. The apparent $V_{\text{max}}$ is not influenced by the structure of the OS-PP-Dol donor, as revealed by a shared $I/V$ intercept, when the oligosaccharide donors are present in fourfold excess relative to the apparent $K_m$ for the donor substrate (Fig. 6 A).

### Discussion

#### Donor substrate selection of nonglucosylated oligosaccharides

GN$_2$-PP-Dol is the smallest oligosaccharide donor that is an effective substrate for the yeast OST (Sharma et al., 1981; Bause et al., 1995; Gibbs and Coward, 1999). The 2′N-acetyl modification on the first saccharide is critical for catalysis, whereas the 2′N-acetyl modification on the second residue is important for substrate recognition (Tai and Imperiali, 2001). Efficient N-glycosylation by the OST from higher eukaryotes is also dependent on the terminal glucose residue on the A antennae of the oligosaccharide (Turco et al., 1977; Trimble et al., 1980). As the OS-PP-Dol donors synthesized by many protists and the fungi C. neoformans lack glucose residues, one might predict that the OST from these organisms would only recognize the GlcNAc$_2$ core of the donor substrate. However, donor substrate competition experiments demonstrate that the in vivo oligosaccharide donor for T. vaginalis, E. histolytica, T. cruzi, and C. neoformans is a preferred substrate relative to certain larger and/or smaller OS-PP-Dol compounds. To our knowledge, this is the first evidence that oligosaccharide donor substrate selection is not restricted to organisms that synthesize the trigramosylated oligosaccharide donor. In all four cases, preferential utilization of the in vivo donor is less stringent than that observed for the S. cerevisiae or mammalian OST both in terms of the size range of compounds that are optimal in vitro substrates and the fold selection of the in vivo donor substrate relative to nonoptimal donors.

The predicted one-subunit OST from T. cruzi utilizes larger OS-PP-Dol compounds, including the in vivo donor M5GN$_2$-PP-Dol in preference to M5GN$_2$-PP-Dol. The latter compound is one of four luminal OS-PP-Dol assembly intermediates that could compete in vivo with M5GN$_2$-PP-Dol as a donor substrate. The observed two- to threefold more rapid in vitro transfer of M5GN$_2$-PP-Dol than M5GN$_2$-PP-Dol appears to be sufficient to ensure that small assembly intermediates are rarely used in vivo, in part because M5GN$_2$-PP-Dol is more abundant in the T. cruzi ER than the luminally oriented (M$_3$sGN$_2$-PP-Dol) assembly intermediates (Parodi and Quesada-Alue, 1982). The presence of a terminal α-1,2–linked mannose residue on the B or C antennae appears to be important for preferential utilization of M5GN$_2$-PP-Dol by the T. cruzi OST, as revealed by the relative transfer rates of M5GN$_2$-PP-Dol isomer classes (Fig. 3) and by the reduced utilization of M5GN$_2$-PP-Dol relative to M5GN$_2$-PP-Dol. In vivo transfer of an assembly intermediate may be deleterious, as protein-linked high-mannose oligosaccharides that lack the terminal mannose residue on the B antennae (M8B isomer) or C antennae (M8C isomer) are less efficiently glucosylated by the UDP-glucose glycoprotein.

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**Figure 5.** Kinetic parameters for the oligosaccharide donor substrate. OST activity was assayed using a constant concentration of the acceptor tripeptide substrate [10 μM in A, B, and D and 15 μM in C] and variable concentrations of M$_5$GN$_2$-PP-Dol [A–C] or M$_5$GN$_2$-PP-Dol [D]. [A, C, and D] Lineweaver-Burk plots (1/OST activity versus 1/[OS-PP-Dol]) for the T. vaginalis [A; $K_m = 0.22 \mu M$, E. histolytica [C; $K_m = 0.72 \mu M$], or T. cruzi [D; $K_m = 0.49 \mu M$] OST were linear. [B] An Eadie-Hofstee plot (OST activity vs. OST activity/[OS-PP-Dol]) for the T. vaginalis ($K_m = 0.19 \mu M$) OST was linear. The inset shows an Eadie-Hofstee plot for the S. cerevisiae OST using M$_5$GN$_2$-PP-Dol as the donor substrate.

**Figure 6.** Kinetic parameters for the tripeptide acceptor substrate. OST activity was assayed using a constant concentration of the OS-PP-Dol donor (1 μM M$_5$GN$_2$-PP-Dol or G$_3$M$_5$GN$_2$-PP-Dol in A and 0.8 μM M$_5$GN$_2$-PP-Dol or M$_5$GN$_2$-PP-Dol in B) and increasing concentrations of acceptor tripeptide substrate. [A] Lineweaver-Burk plots for the T. vaginalis OST yielded apparent $K_m$ values of 17 μM (M$_5$GN$_2$-PP-Dol donor) and 53 μM (G$_3$M$_5$GN$_2$-PP-Dol) for the acceptor tripeptide. [B] Lineweaver-Burk plots for the T. cruzi OST yielded apparent $K_m$ values of 2.3 μM (M$_5$GN$_2$-PP-Dol donor) and 6.9 μM (M$_5$GN$_2$-PP-Dol) for the acceptor tripeptide.
glucosyltransferase (UGGT; Trombetta and Parodi, 2003). UGGT, which was first detected in T. cruzi, serves as the folding sensor for the glycoprotein quality-control pathway in the ER (Caramelo et al., 2003).

The predicted four-subunit OSTs from T. vaginalis and E. histolytica (Fig. 1) transfer the in vivo donor (M₅GN₂-PP-Dol) at the same rate as other OS-PP-Dol compounds that lack glucose residues (M₃GN₂-PP-Dol), including M₃GN₂-PP-Dol iso-

mers that lack one or more mannose residues on the A antennae. Because synthesis of the M₅GN₂-PP-Dol donor is completed on the cytoplasmic face of the rough ER, the T. vaginalis and E. histolytica OST do not need to discriminate between luminally oriented M₅GN₂-PP-Dol and cytoplasmically oriented OS-PP-Dol assembly intermediates. Consequently, M₅GN₂-

NYT is the major glycopeptide synthesized in vitro when an acceptor tripeptide is incubated with intact T. vaginalis or E. histolytica membranes (Samuelson et al., 2005) despite the lack of a mechanism to discriminate against underassembled oligosaccharide donors. We propose that the STT3 active-site subunit of the OST has evolved to have a catalytic site that is optimal for the in vivo oligosaccharide. For T. vaginalis, E. histolytica, and C. neoformans, the proposed loss of genes that encode the ALG glucosyltransferases (ALG6, -8, and -10; Samuelson et al., 2005) has apparently been accompanied by compensatory alterations in the STT3 structure that are optimal for an oligosaccharide donor with an A antennae that lacks all three glucose residues.

The predicted seven-subunit C. neoformans OST transfers the larger mannosylated OS-PP-Dol donors (M₇-9GN₂-PP-Dol) more rapidly than smaller assembly intermediates or G₃M₉GN₂-

PP-Dol. Utilization of the fully assembled in vivo donor in preference to luminally exposed OS-PP-Dol assembly intermediates may be a shared property of the OST in organisms that synthesize donors larger than M₅GN₂-PP-Dol. The relatively modest (~1.5-fold) preference for M₅GN₂-PP-Dol relative to biosynthetic M₅GN₂-PP-Dol leads to selective synthesis of M₅GN₂-

NYT when the acceptor tripeptide is incubated with intact C. neoformans membranes (Samuelson et al., 2005).

Kinetic analysis of the T. cruzi and T. vaginalis OST revealed that oligosaccharide structure-mediated modulation of acceptor substrate binding affinity is a conserved property of the eukaryotic OST that can be ascribed to the STT3 active site. The threefold reduction in acceptor substrate binding affinity readily accounts for the reduced transfer of nonoptimal donors when the acceptor tripeptide is present at subsaturating levels. Future studies will address the order of substrate binding to the one- and four-subunit OSTs that are predicted for T. cruzi and E. histolytica. One objective of these experiments will be to determine whether the subunit composition of protist complexes matches the bioinformatic predictions.

Candidate subunits for the regulatory OS-PP-Dol binding site

Cooperative OS-PP-Dol binding by the S. cerevisiae OST is not explained by dimerization of heteroooligomers, as communoprecipitation experiments using yeast strains that express STT3-

HA₃ and STT3-His₅FLAG₁ from chromosomal loci did not reveal higher order OST oligomers (Karaoglu et al., 2001). Potential explanations for the discrepancy between a recent report describing dimeric assembly of the yeast OST complex (Chavan et al., 2006) and our previous conclusions are being explored. Cooperative OS-PP-Dol binding is not explained by separate but interacting binding sites for the chitinobiose core of G₃M₉GN₂-PP-Dol and the terminal glucose residue, because cooperative binding by the yeast or canine OST is not dependent on the presence of glucose residues on the oligosaccharide donor, as confirmed here using M₃GN₂-PP-Dol as a donor substrate. Instead, our results indicate that cooperative donor substrate binding is diagnostic of a regulatory OS-PP-Dol binding site that is primarily responsible for the highly selective utilization of the G₃M₉GN₂-PP-Dol donor (Karaoglu et al., 2001; Kelleher et al., 2003).

Based on a kinetic analysis of canine OST isoforms, we proposed that the regulatory OS-PP-Dol binding site is not located on the catalytic subunit (STT3A or -B), but is instead provided by one or more of the shared noncatalytic subunits. Support for this hypothesis has now been provided by recent experiments showing that a T. cruzi STT3 can assemble with the noncatalytic yeast OST subunits and, upon doing so, mediate selective utilization of G₃M₉GN₂-PP-Dol as the donor substrate both in vitro and in vivo (Castro et al., 2006).

One objective of this study was to determine whether protist OSTs use a regulatory OS-PP-Dol binding site to select the in vivo oligosaccharide donor. Unlike the S. cerevisiae and Canis familiaris OST, the predicted one-subunit OST from T. cruzi (STT3) and the predicted four-subunit OSTs from E. histolytica and T. vaginalis (STT3-OST1-OST2-WBP1) do not bind OS-

PP-Dol in a cooperative manner; hence, the OST from these organisms lacks the regulatory OS-PP-Dol binding site. The simplest interpretation of this observation is that the regulatory OS-PP-Dol binding arose as additional subunits were acquired during evolution of the eukaryotic OST. The IAP and TUSC3 (N33) proteins dissociate from the canine OST during purification, so these OST3/OST6 family members are not candidates for the regulatory OS-PP-Dol binding site. OST4 and -5 can be discounted based on structural considerations because neither of these polypeptides has more than a few residues exposed to the lumen of the ER (Fig. 1). Therefore, cooperative OS-PP-Dol binding by the yeast or vertebrate OST correlates with the presence of a Swp1p/ribophorin II subunit in the OST complex. Extensive biochemical and genetic evidence supports direct interactions between Wbp1, Swp1p, and Os2p (te Heesen et al., 1993; Silberstein et al., 1995), as well as between their respective mammalian homologues, OST48, ribophorin II, and DAD1 (Fu et al., 1997; Kelleher and Gilmore, 1997). We hypothesize that the regulatory OS-PP-Dol binding site is located on the Swp1p–Wbp1p–Os2p subcomplex. Interestingly, OS-

PP-Dol protects a critical cysteine residue in Wbp1p from modification by a cysteine-directed protein modification reagent (Pathak et al., 1995). A role for the Swp1p–Wbp1p–Os2p subcomplex as the regulatory OS-PP-Dol binding site might help explain why expression of each of these subunits is essential for viability of S. cerevisiae (te Heesen et al., 1992, 1993; Silberstein et al., 1995). With the exception of C. neoformans,
there is a strong correlation between organisms that assemble a glucosylated oligosaccharide donor (either G, M, GN -PP-Dol or G, M, GN -PP-Dol) and organisms that express or are predicted to express a Swpl/ribophorin II homologue (Samuelson et al., 2005; Kelleher and Gilmore, 2006).

Materials and methods

Preparation of detergent-extracted membranes and the S. cerevisiae OST
Trophozoites of E. histolytica strain HM1:IMSS were grown axenically (in the absence of bacteria or other cells) in TFI medium supplemented with 10% heat-inactivated adult bovine serum at 37°C. Axenic cultures of T. vaginalis strain G3 were maintained in TFM medium supplemented with 10% heat-inactivated horse serum at 37°C. Axenic cultures of T. cruzi epimastigotes (strain Y) were grown in the UT medium supplemented with hemin and 10% heat-inactivated fetal calf serum at 25°C. C. neoformans strain B3501, maintained on YPD plates, was grown in YPD broth for 20 h at 30°C.

Whole cells were collected by centrifugation and resuspended in 10 mM Hepes, pH 7.4, 20 mM MgCl2, and 1x protease inhibitor cocktail (PIC; as defined by Kelleher et al., 1992). E. histolytica, T. vaginalis, or T. cruzi cells were homogenized using 50 strokes of a Teflon-glass homogenizer. The C. neoformans cell suspension was mixed with an equal volume of glass beads and vortexed extensively (200 s bursts). Total membrane fractions were collected by a 30-min centrifugation of the cell homogenate at 267,000 g, using a rotor (TLA 100.4; Beckman Coulter). The membrane pellets were solubilized in 1.5% digitonin, 20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM DTT, and 1x PIC at a membrane concentration of 2 eq/μl (1 eq/μl = 50 A280 in 1% SDS). The detergent extracts were clarified by a 5-min centrifugation at 66,600 g, using the rotor. The S. cerevisiae OST was purified from an epitope-tagged (6xHisFLAG-OST1) yeast strain as described previously (Karaoglu et al., 2001).

OST assays
Detergent extracts of the E. histolytica, T. vaginalis, T. cruzi, and C. neoformans membranes were diluted fourfold with 20 mM Tris-Cl, pH 7.4, 1 mM MgCl2, 1 mM MnCl2, 1 mM DTT, and 1x PIC. 5 μl aliquots of the 4× diluted soluble extracts were assayed for OST activity in a total volume of 100 μl as described previously (Kelleher and Gilmore, 1997), using N-acetylmannohexosaminide (ManHexNAc) as the acceptor substrate and either structurally homogeneous OS-PP-Dol compounds or a previously described heterogeneous bovine pancreas OS-PP-Dol pool (Kelleher et al., 2001) as the donor substrate. OST assays were supplemented with 1.4 mM deoxynojirimycin, 1.4 mM mannojirimycin, and 1.4 mM swainsonine to inhibit glucosidases and mannosidases. Glycopeptide products from OST assays were isolated with ConA Sepharose and quantified by gamma counting.

Structurally homogeneous G, M, GN -PP-Dol, M, GN -PP-Dol, and an enriched G, M, GN -PP-Dol preparation were purified as described previously (Kelleher et al., 2001) from porcine pancreas (G, M, GN -PP-Dol and M, GN -PP-Dol) or an αL3α yeast strain (M, GN -PP-Dol), or an αL3δ yeast strain (M, GN -PP-Dol). The concentration and composition of OS-PP-Dol samples was determined from the yield and oligosaccharide distribution of radiolabeled glycopeptides obtained in the OST endpoint assay (Kelleher et al., 2001). In brief, 12–15 pmol of OS-PP-Dol was incubated with 60 fmol of purified OST assays to prepare M, GN -glycopeptides using the heterogeneous OS-PP-Dol library were designed to ensure that <10% of the total M, GN -PP-Dol was converted to M, GN -NYP. HPLC fractions corresponding to M, GN -NYP were collected and resuspended in 50 μl of 1× reaction buffer supplied by the manufacturer (Prozyme) and incubated for 18 h at 37°C with 0.33 mM α-1,2 mannosidase. The Savant-dried glycopeptide digestion products were dissolved in 500 μl HPLC buffer A and resolved by HPLC as described in the preceding paragraph.

Analysis of kinetic data
The kinetic parameters for the tripeptide acceptor and oligosaccharide donor for the T. vaginalis, T. cruzi, and E. histolytica enzymes were determined by a nonlinear least-squares fit of the kinetic data to the Michaelis-Menten equation and by linear least-squares fits of Lineweaver-Burk plots or Eadie-Hofstee plots. The kinetic parameters for the dolichol-oligosaccharide donor for the S. cerevisiae enzyme were obtained using a nonlinear least-squares fit of the kinetic data to equations for a substrate activated enzyme as described previously (Karaoglu et al., 2001). Kaleidagraph (Synergy Software) was used for curve fitting.

Online supplemental material
Fig. S1 shows the oligosaccharide composition analysis of the OS-PP-Dol library used for the experiments in Fig. 2 and Fig. 3. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200611079/DCT1.

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References

Bause, E., W. Breuer, and S. Peters. 1995. Investigation of the active site of oligosaccharidotransferase from pig liver using synthetic tripeptides as tools. Biochem. J. 312:979–985.

Bosch, M., S. Trombetta, U. Engstrom, and A.J. Parodi. 1988. Characterization of dolichol diphasphate oligosaccharide: protein oligosaccharidotransferase and glycoprotein-processing glucosidases occurring in trypanosomatid protozoa. J. Biol. Chem. 263:17360–17365.

Breuer, W., and E. Bause. 1995. Oligosaccharidotransferase is a constitutive component of an oligomeric protein complex from pig liver endoplasmic reticulum. Eur. J. Biochem. 228:689–696.

Burd, P., and M. Aebi. 1999. The dolichol pathway of N-linked glycosylation. Biochim. Biophys. Acta. 1426:239–257.

Caramelo, J.J., O.A. Castro, L.G. Alonso, G. De Prat-Gay, and A.J. Parodi. 2003. UDP-Glc:glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates. Proc. Natl. Acad. Sci. USA. 100:86–91.

Castro, O., F. Movsichoff, and A.J. Parodi. 2006. Preferential transfer of the complete glycan is determined by the oligosaccharidotransferase complex and not by the catalytic subunit. Proc. Natl. Acad. Sci. USA. 103:14756–14760.

Chavan, M., Z. Chen, G. Li, H. Schindelin, W.J. Lennarz, and H. Li. 2006. Dimeric organization of the yeast oligosaccharidotransferase complex. Proc. Natl. Acad. Sci. USA. 103:8947–8952.

de la Canal, L., and A.J. Parodi. 1987. Synthesis of dolichol derivatives in trypanosomatids. Characterization of enzymatic patterns. J. Biol. Chem. 262:11128–11133.

Freeze, H.H., and M. Aebi. 2005. Altered glycan structures: the molecular basis of congenital disorders of glycosylation. Curr. Opin. Struct. Biol. 15:490–498.

Fu, J., M. Ren, and G. Kreibich. 1997. Interactions among subunits of the oligosaccharidotransferase complex. J. Biol. Chem. 272:29687–29692.
Gavel, Y., and G. Von Heijne. 1990. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. Protein Eng. 3:433–442.

Gibbs, B.S., and J.K. Coward. 1999. Dolichylphosphate oligosaccharides: large scale isolation and evaluation as oligosaccharyltransferase substrates. Biochim. Biophys. Acta 1425:169–187.

Helenius, A., and M. Aebi. 2004. Ways of N-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem. 73:1019–1049.

Helenius, J., D.T. Ng, C.L. Marolda, P. Walter, M.A. Valvano, and M. Aebi. 1992. The yeast Wbp1 is essential for oligosaccharyl transferase activity in vivo and in vitro. Proc. Natl. Acad. Sci. USA. 89:4114–4118.

Kelleher, D.J., and R. Gilmore. 1992. The STT3 subunit of the oligosaccharyltransferase complex. J. Biol. Chem. 267:11892–11895.

Kelleher, D.J., D. Karaoglu, and R. Gilmore. 2001. Large-scale isolation of dolichol-linked oligosaccharides with homogeneous oligosaccharide structures: determination of steady-state dolichol-linked oligosaccharide compositions. Glycobiology. 11:321–333.

Kelleher, D.J., D. Karaoglu, E.C. Mandon, and R. Gilmore. 2003. Oligosaccharyltransferase isoforms that contain different catalytic STT3 subunits have distinct enzymatic properties. Mol. Cell. 12:101–111.

Koiwa, H., F. Li, M.G. McCully, I. Mendoza, N. Koizumi, Y. Manabe, Y. Nakagawa, J. Zhu, A. Rus, J.M. Pardo, et al. 2003. The STT3a subunit isoform of the Arabidopsis oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. Plant Cell. 15:2273–2284.

Mellis, S.J., I. Mendoza, N. Koizumi, Y. Manabe, Y. Nakagawa, H. Koiwa, K. Hiramatsu, A. Pitha, Y. Hara, Y. Nakagawa, et al. 2003. The STT3a subunit isoform of the Arabidopsis oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. Plant Cell. 15:2273–2284.

Parodi, A.J. 1993. N-glycosylation in trypanosomatid protozoa. Glycobiology. 3:193–199.

Parodi, A.J., and L. A. Quesada-Allue. 1982. Protein glycosylation in Trypanosoma cruzi. I. Characterization of dolichol-bound monosaccharides and oligosaccharides synthesized in vivo. J. Biol. Chem. 257:7637–7640.

Pathak, R., T.L. Hendrickson, and B. Imperiali. 1995. Sulphydryl modification of the yeast Wbp1p inhibits oligosaccharyl transferase activity. Biochemistry. 34:4179–4185.

Samuelson, J., S. Banerjee, P. Magnelli, J. Cui, D.J. Kelleher, R. Gilmore, and P.W. Robbins. 2005. The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. Proc. Natl. Acad. Sci. USA. 102:1548–1553.

Sharma, C.B., L. Lehle, and W. Tanner. 1981. N-glycosylation of yeast proteins. Characterization of the solubilized oligosaccharyltransferase. Eur. J. Biochem. 116:101–108.

Silberstein, S., P.G. Collins, D.J. Kelleher, and R. Gilmore. 1995. The essential OST2 gene encodes the 16-kD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms. J. Cell Biol. 131:371–383.

Spirig, U., M. Glavas, D. Bodmer, G. Reiss, P. Burda, V. Lippuner, S. te Heesen, and M. Aebi. 1997. The STT3 protein is a component of the yeast oligosaccharyltransferase complex. Mol. Gen. Genet. 256:628–637.

Tai, W.V., and B. Imperiali. 2001. Substrate specificity of the glycosyl donor for oligosaccharyl transferase. J. Org. Chem. 66:6217–6228.

Te Heesen, S., B. Janetzky, L. Lehle, and M. Aebi. 1992. The yeast WBP1 is essential for oligosaccharyltransferase activity in vivo and in vitro. EMBO J. 11:2071–2075.

Te Heesen, S., R. Knauer, L. Lehle, and M. Aebi. 1993. Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity. EMBO J. 12:279–284.

Trimble, R.B., J.C. Byrd, and F. Maley. 1980. Effect of glucosylation of lipid intermediates on oligosaccharyl transfer in solubilized microsomes from Saccharomyces cerevisiae. J. Biol. Chem. 255:11892–11895.