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

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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 G₃M₉GN₂-PP-Dol). Synthesis of the dolichol-linked oligosaccharide 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 Aebi, 1999). Man₅GlcNAc₂-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, Entamoeba histolytica, Trichomonas vaginalis, Cryptococcus neoformans, and Saccharomyces cerevisiae) use structurally homogeneous dolichol-linked oligosaccharides 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.

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Abbreviations used in this paper: ALG, asparagine-linked glycosylation; HPLC, high-pressure liquid chromatography; OS-NYT, glycosylated tripeptide; OS-PP-Dol, dolichol-linked oligosaccharide; OST, oligosaccharyltransferase; PIC, protease inhibitor cocktail.

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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* and *Cryptococcus neoformans* synthesize 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., 2006). The genomes of *G. lamblia* and the kinetoplastids *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 G6M3GN2-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 G6M3GN2-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 G6M3GN2-PP-Dol (for review see Freeze and Aebi, 2005).

Preferential utilization of G6M3GN2-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-Dol transfer the in vivo donor in preference to OS-PP-Dol assembly intermediates or G6M3GN2-PP-Dol? Previous studies indicate that the *T. cruzi* OST transfers glucosylated (G6M3GN2-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 components assembled with a shared set of noncatalytic subunits (ribophorin I [Ost1 homologue], ribophorin II [Swp1], OST48 [Wbp1], DAD1 [Ost2], and TUSC3 or IAP [Ost3 -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 kinetoplastids *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 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.

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 luminaally 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.

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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 M₃GN₂-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-NYT was the most abundant product when the purified *S. cerevisiae* OST was assayed (Fig. 2 A). In contrast, G₃M₉GN₂-NYT 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 (M₅GN₂-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 M₃GN₂-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 (M₃GN₂-PP-Dol) were transferred less rapidly (Fig. 2 F). The *C. neoformans* OST showed preferential utilization of the in vivo donor (M₃GN₂-PP-Dol) relative to assembly intermediates (M₃,M₅GN₂-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., M₅GN₂-NYT) 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 M₅GN₂-PP-Dol (Fig. 3 A, compound a) could yield seven M₃GN₂-PP-Dol isomers (Fig. 3 A, compounds c–i) that differ from biosynthetic M₅GN₂-PP-Dol (Fig. 3 A, compound b). Biosynthetic M₅GN₂-PP-Dol (Fig. 3 A, compound b) can be readily distinguished from these other isomers by digestion with α-1,2 mannosidase. Resolution of the predicted one- and four-subunit protist OST complexes.
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 M3GN2-NYT (right). The M5GN2-NYT (M3) peak is derived from isomer b, the M3GN2-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-PP-Dol synthesized by the S. cerevisiae (Sc), T. cruzi (Tc), E. histolytica (Eh), and T. vaginalis (Tv) OST. 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.

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 M3-9GN2-PP-Dol relative to M5-9GN2-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 G3MGN2-PP-Dol in preference to both nonglicosylated 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 nonglicosylated
be recognized by the OST in these organisms. To test this hypothesis, an additional competition experiment was performed using a mixture of purified M₅GN₂-PP-Dol and an enriched sample of G₂M₉GN₂-PP-Dol. The G₃M₉GN₂-PP-Dol preparation contains G₁M₉GN₂-PP-Dol as a minor component. Glycopeptide products synthesized by the S. cerevisiae and T. vaginalis OST 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 yielded Kₘ 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 Kₘ values 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 (Karacolgu et al., 2001; Kelleher et al., 2003). The nonoptimal donor substrate (G,M,G,N2-PP-Dol for T. vaginalis and M,G,N2-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 Vmax 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 Km for the donor substrate (Fig. 6 A).

Discussion

Donor substrate selection of nonglucosylated oligosaccharides

GN2-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 GlcNAc2 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 trilglycosylated oligosaccharide donor. In all four cases, preferential utilization of the in vivo donor is less stringent than that observed for the S. cerevisiae OST 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 M,G,N2-PP-Dol in preference to M,G,N2-PP-Dol. The latter compound is one of four luminal OS-PP-Dol assembly intermediates that could compete in vivo with M,G,N2-PP-Dol as a donor substrate. The observed two- to threefold more rapid in vitro transfer of M,G,N2-PP-Dol than M,G,N2-PP-Dol appears to be sufficient to ensure that small assembly intermediates are rarely used in vivo, in part because M,G,N2-PP-Dol is more abundant in the T. cruzi ER than the luminally oriented (M,s,G,N2-PP-Dol) assembly intermediates (Parodi and Quesada-Allue, 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 M,s,G,N2-PP-Dol by the T. cruzi OST, as revealed by the relative transfer rates of M,s,G,N2-PP-Dol isomer classes (Fig. 3) and by the reduced utilization of M,s,G,N2-PP-Dol relative to M,G,N2-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
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 (M₇GN₂-PP-Dol), including M₃GN₂-PP-Dol isomers that lack one or more mannose residues on the A antennae. Because synthesis of the M₃GN₂-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, M₃GN₂-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 (M₇GN₂-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 M₃GN₂-PP-Dol. The relatively modest (~1.5-fold) preference for M₃GN₂-PP-Dol relative to biosynthetic M₇GN₂-PP-Dol leads to selective synthesis of M₃GN₂-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 protost 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 explained by dimerization of hetero-oligomers, as coimmunoprecipitation experiments using yeast strains that express STT3-HA₃ and STT3-His₉-FLAG₁ 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 chitobiose 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 M₅GN₂-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 protost 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–Wbp1–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 \( \text{G}_2\text{M}_9\text{GN}_2\)-PP-Dol or \( \text{G}_3\text{M}_5\text{GN}_2\)-PP-Dol) and organisms that express or are predicted to express a \( \alpha\)-1,2 mannosidase.

**Materials and methods**

**Preparation of detergent-extracted membranes and the \( \text{S. cerevisiae} \) OST**

Trophozoites of \( \text{E. histolytica} \) strain HM1:IMSS were grown axenically (in the absence of bacteria or other cells) in TYI medium supplemented with 10% heat-inactivated adult bovine serum at 37°C. Axenic cultures of \( \text{T. vaginalis} \) strain G3 were maintained in TYM medium supplemented with 10% heat-inactivated horse serum at 37°C. Axenic cultures of \( \text{C. neoformans} \) strain Y were grown in the UT medium supplemented with hemin and 10% heat-inactivated fetal calf serum at 35°C. \( \text{C. neoformans} \) 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, 25 mM NaCl, 10 mM MgCl₂, and 1 mM protease inhibitor cocktail (PIC; as defined by Kelleher et al., 1992). \( \text{E. histolytica} \), \( \text{T. vaginalis} \), or \( \text{C. neoformans} \) cells were homogenized using 50 strokes of a Teflon-glass homogenizer. The \( \text{C. neoformans} \) cell suspension was filtered through 3.5×0.8-μm membranes and stored at 4°C. Homogenates of \( \text{T. vaginalis} \), \( \text{T. cruzi} \), or \( \text{C. neoformans} \) cells were homogenized using 50 strokes of a Dounce homogenizer. The detergent extracts were clarified by a 5-min centrifugation at 4°C, 60,000 gₑ using the rotor. The \( \text{S. cerevisiae} \) OST was purified from an epitope-tagged (6×HisFLAG-OST1) yeast strain as described previously (Karaoglu et al., 2001).

**OST assays**

Detergent extracts of the \( \text{E. histolytica} \), \( \text{T. vaginalis} \), \( \text{T. cruzi} \), and \( \text{C. neoformans} \) membranes were diluted fourfold with 20 mM Tris·Cl, pH 7.4, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM DT, and 1× 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 \( \text{N-acetyl}-\alpha\text{-Asn}-[\text{Tyr}^{125}\text{I}]-\text{Thr}-\text{NH}_2 \) as the acceptor substrate and either structurally homogeneous OS-PP-Dol or 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 deoxyojirimycin, 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 \( \text{G}_2\text{M}_9\text{GN}_2\)-PP-Dol, \( \text{M}_5\text{GN}_2\)-PP-Dol, and an enriched \( \text{G}_2\text{M}_9\text{GN}_2\) component of an oligomeric protein complex from pig liver endoplasmic reticulum. Eur. J. Biochem. 263:17360–17365.


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**References**


