Molecular characterization of the cis-prenyltransferase of Giardia lamblia

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Giardia lamblia, the protist that causes diarrhea, makes an Asn-linked-glycan (N-glycan) precursor that contains just two sugars (GlcNAc₂) attached by a pyrophosphate linkage to a polyisoprenoid lipid. Because the candidate cis-prenyltransferase of Giardia appears to be more similar to bacterial enzymes than to those of most eukaryotes and because Giardia is missing a candidate dolichol kinase (ortholog to S. cerevisiae SEC59 gene product), we wondered whether Giardia synthesizes dolichol pyrophosphate (Dol-P), which is used to make an unusually short N-glycan precursor (dolichol pyrophosphate-GlcNAc₂) and to make dolichol phosphate mannose (Dol-P-Man) (Heleiniius and Aebi 2004; Samuelson et al. 2005). While many eukaryotes use Dol-P-Man to make N-glycan precursors, O-linked glycans and glycosylphosphatidylinositol (GPI) anchors, Giardia only uses Dol-P-Man to make GPI anchors (Das et al. 1991; Orlean 1990; Samuelson et al. 2005).

Eukaryotic cis-prenyltransferases, which are encoded by RER2 and SRT1 genes of Saccharomyces cerevisiae, use farnesyl pyrophosphate (FPP) and numerous isopentenyl pyrophosphates (IPP) to make dehydrodolichyl pyrophosphate (Dedol-PP) (Figure 1) (Grabińska and Palamarczyk 2002; Sato et al. 1999, 2001). Dominant Dedol-PPs contain 11 isoprene units (e.g. Plasmodium, Leishmania and Trypanosoma), 16 isoprene units (e.g. S. cerevisiae Rer2p product and Trichomonas) or 19 isoprene units (e.g. S. cerevisiae Srt1p product and human) (Arruda et al. 2005; D’Alexandri et al. 2006; Grabińska et al. 2008; Löw et al. 1991; Swiezewska and Danikiewicz 2005). Dedol-PPs are subsequently dephosphorylated and then reduced to dolichol by a saturase, which has not yet been molecularly characterized (Fujii et al. 1982; Sagami et al. 1993). A cytidine triphosphate (CTP)-dependent dolichol kinase, encoded by the SEC59 gene of S. cerevisiae, converts dolichol to Dol-P (Heller et al. 1992). In bacteria, undecaprenol kinase activity is adenosine triphosphate (ATP)-dependent (Kalin and Allen 1979; Lis and Kuramitsu 2003).

Eubacteria and archaea have a cis-prenyltransferase, which makes Dedol-PP that contains 11 isoprene units (Kato et al. 1999). Like eukaryotes, archaea have a saturase that converts dehydrodolichol (Dedol) to dolichol (Burda and Aebi 1999). In contrast, eubacterial Dedol, which is used to make precursors for peptidoglycans and lipopolysaccharides, remains unsaturated (Touzé et al. 2008).

Because the candidate cis-prenyltransferase of Giardia appears to be more similar to bacterial enzymes than to most eukaryotic enzymes (see Results) and because Giardia is missing a candidate dolichol kinase (Sec59p homolog), we wondered whether Giardia synthesizes Dol-P in a manner similar to the rest of eukaryotes (Figure 1). For example, does the Giardia cis-prenyltransferase make Dedols with 11 isoprene units like those of bacteria? Does Giardia have an alternative saturase (marked in green in Figure 1), which converts Dedol-P to dolichol-P? Or does Giardia, like eubacteria,
use Dedol-P for synthesis of polyprenol-linked glycans (e.g. Dedol-P-Man and Dedol-PP-GlcNAc) (alternative DPM1 and ALG7 gene products marked in blue in Figure 1)? Do Giardia membranes have CTP-dependent dolichol kinase activity, even though the parasite appears to lack a SEC59 ortholog (orange in Figure 1)?

Fig. 1. Mapping of predicted Giardia enzymes onto the eukaryotic synthetic pathway for dolichol phosphate (Dol-P). Orthologs to Saccharomyces enzymes, which are present in Giardia (e.g. Rer2/Srt1, Alg7 and Dpm1) are marked in red. Saccharomyces Sec59, which appears to be absent in Giardia, is marked in orange. Enzymes that have not been molecularly characterized and so are not identified by searches with BLASTP (e.g. Dedol saturase) are marked in gray. Two alternative pathways for Dol-P synthesis in Giardia, which are tested by experimentation here, are the conversion of Dedol-P to Dol-P (marked in green) and use of Dedol-P rather than Dol-P for synthesis of N-glycans and GPI anchors (marked in blue).

Fig. 2. Thin layer chromatography (TLC) shows the Giardia undecaprenyl pyrophosphate synthase (Gi-UPPS), which closely resembles the bacterial UPPS (Supplement Figure 1 and see Figure 4 below) that makes a dominant polyrenyl pyrophosphate with 11 isoprene units (Dedol-11). (A) In the left lane, Giardia membranes incubated with radiolabeled IPP make Dedol-11. Faster running label represents short chain prenols. In the right lane, membranes from a Saccharomyces rer2Δ, srt1Δ double-mutant expressing Gi-UPPS also make Dedol-11 when incubated with radiolabeled IPP. (B) Recombinant Gi-UPPS, which was purified from transformed E. coli, makes polyrenyl pyrophosphate with 11 isoprene units (Dedol-11-PP), which is converted to Dedol-11-P by treatment with bacA, which is an undecaprenyl pyrophosphate phosphatase. In both A and B, the origin is at the bottom of the TLC plate. In A the resolving zone is shown, while in B the origin is also shown.
Results

Giardia makes in vivo a saturated polyprenol (dolichol) containing 11 (major) and 12 (minor) isoprene units.

The starting point for these studies was a characterization of the cis-prenyltransferase activity of membranes isolated from cultured Giardia. Giardia membranes make an unsaturated polyprenyl (Dedol-11), which contains 11 isoprene units when incubated with radiolabeled IPP and exogenous FPP (Figure 2). Dedol-PP phosphatase and Dedol-P phosphatase activities are inferred, because Dedol is produced by the Giardia membranes. These results are consistent with the presence of candidate cis-prenyltransferase in Giardia (see next section) (Marchler-Bauer et al. 2005).
Mass spectroscopy of polyprenols of cultured *Giardia* also demonstrated dolichols with 11 and 12 isoprene units (Dol-11 and Dol-12) (Figure 3A). Using the same methods, dolichols with 14 to 17 isoprene units (Dol-14 to Dol-17) were identified in *Saccharomyces*. The presence of Dedol-11 and Dol-11 within *Giardia* argues against alternative pathways for synthesis of Dol-P, which are marked in green and blue in Figure 1. Instead it appears that *Giardia* is synthesizing Dol-P in the same way as other eukaryotes, even though *Giardia* is missing a dolichol kinase candidate (*SEC59* ortholog marked in orange in Figure 1) and the *Giardia cis*-prenyltransferase resembles those of bacteria (next section).

*Giardia cis*-prenyltransferase resembles those of eubacteria and *Trypanosoma*

The predicted *Giardia cis*-prenyltransferase (encoded by *GiardiaDB 50803_15256*) is 265-amino acids long and shows a 42% identity and a 58% similarity to the *Escherichia coli* undecaprenyl pyrophosphate synthase (Ec-UPPS), which has been crystallized (see Supplemental Figure 1) (Guo et al. 2005). The *Giardia cis*-prenyltransferase, which we will refer to as Gl-UPPS because the protist synthesizes Dedol-PP with 11 isoprene units (Figure 2), contains five conserved domains that have been identified in other *cis*-prenyltransferases.

Phylogenetic analysis of representative eukaryotic and prokaryotic *cis*-prenyltransferases revealed three important findings (Figure 4). First, the vast majority of eukaryotic enzymes are present in a group (Clade 1), which is distinct from the group (Clade 2) that includes eubacteria and archaea. Second, *Giardia*, *Trypanosoma, Leishmania* and the chloroplast enzyme are also present with bacteria in Clade 2. The presence of a small number of eukaryotes in the bacterial clade is suggestive of LGT, which is a major force in the evolution of *Giardia* (Andersson et al. 2003; Franzén et al. 2009; Morrison et al. 2007). However, the same results could also be explained by secondary loss of the Clade 2 *cis*-prenyltransferase from the majority of eukaryotes. Previously, the diversity in the length of sugars present in N-glycan precursors has been shown to be secondary to loss of ALG enzymes from a common ancestor, which had a complete set (Samuelson et al. 2005).

Third, *Entamoeba* is the only eukaryote examined, which is missing a *cis*-prenyltransferase ortholog. Because the whole genomes of three different *Entamoeba* species have been sepa-
rately sequenced (*E. histolytica*, *E. dispar* and *E. invadens*), the absence of a cis-prenyltransferase ortholog cannot be an artifact of the library construction (Loftus et al. 2005). This result suggests that either *Entamoeba* is synthesizing polyprenyls using an as yet to be identified enzyme, or *Entamoeba* is scavenging polyprenyls from the host.

In a *Saccharomyces rer2Δ, srt1Δ* double-deletion mutant, the *Giardia cis*-prenyltransferase makes a polyprenol containing 11 and 12 isoprene units, and there is no defect in either N-glycan or GPI anchor synthesis.

The *Giardia cis*-prenyltransferase (Gl-UPPS) complements a *Saccharomyces rer2Δ, srt1Δ* double-deletion mutant (Figure 5 and Table 1). The necessity of the Gl-UPPS is shown by the absence of growth of *rer2Δ, srt1Δ* double-deletion mutant when the plasmid containing the Gl-UPPS gene is lost from yeast after treatment with 1% (w/v) 5-fluoroorotic acid (FOA). Gl-UPPS in the *Saccharomyces rer2Δ, srt1Δ* double-deletion mutant makes polyprenols and dolichols, which contain 11 and 12 isoprene units, as described for *Giardia* (Figures 2 and 3, respectively). We could detect no defect in N-glycosylation (using carboxypeptidase Y (CPY) as the reporter) (Supplemental Figure 2) or in GPI anchor synthesis (using glucanosyltransferase encoded by GAS1 (Gas1p) as the reporter) (Supplemental Figure 3). While there is induction of *SRT1* mRNA in the *Saccharomyces rer2Δ* mutant, there is

**Table 1. Yeast strains***

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<tr>
<th>Source</th>
<th>Strain name</th>
<th>Genotype/description</th>
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<tbody>
<tr>
<td>Euroscarf</td>
<td>BY4741</td>
<td>Mat a his3Δ1 leu2Δ1 met15Δ01 ura3Δ1</td>
</tr>
<tr>
<td>Euroscarf</td>
<td>BY4743</td>
<td>MATa/MATα his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ2/lys2Δ2 ura3Δ0/ura3Δ0</td>
</tr>
<tr>
<td>Euroscarf</td>
<td>Y23137</td>
<td>MATa/MATA his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ2/lys2Δ2 ura3Δ0/ura3Δ0 rer2::kanMX4/RER2</td>
</tr>
<tr>
<td>This study</td>
<td>KG219</td>
<td>MATa/MATA his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ2/lys2Δ2 ura3Δ0/ura3Δ0 rer2::kanMX4/RER2</td>
</tr>
<tr>
<td>This study</td>
<td>KG119</td>
<td>As for BY474, rer2::kanMX6 srt1::his3MX6loxP/pNEV–Gl-UPPS</td>
</tr>
<tr>
<td>This study</td>
<td>KG120</td>
<td>As for BY474, rer2::kanMX6 SRT1::his3MX6loxP/pNEV–Gl-UPPS</td>
</tr>
<tr>
<td>Leidich et al. 1994</td>
<td>gpi1</td>
<td>MATα gpi1 ura3-32 his4-917 leu2-3</td>
</tr>
<tr>
<td>Euroscarf</td>
<td>alg5</td>
<td>As for BY474, alg5::kanMX6</td>
</tr>
</tbody>
</table>

**Fig. 5.** Functional complementation of *Saccharomyces rer2Δ, srt1Δ* double-deletion mutant by the *Giardia cis*-prenyltransferase (Gl-UPPS). Wild-type yeast (BY4741), the *rer2Δ* deletion strain, the *rer2Δ* strain expressing Gl-UPPS or the *rer2Δ, srt1Δ* double-deletion strain expressing Gl-UPPS were streaked onto YPD plates or synthetic complete medium containing 1% 5-fluoroorotic acid (FOA). The Ura3 protein, which is expressed from the URA3 marker present in the plasmids, converts FOA to toxic 5-fluorouracil. Wild-type yeast and the single the *rer2Δ* deletion strain are each able to grow in the absence of plasmid, while the *rer2Δ, srt1Δ* double-deletion strain is unable to grow in the absence of the Gl-UPPS expressing plasmid.
Despite the absence of an ortholog to Saccharomyces SEC59, Giardia has a CTP-dependent dolichol kinase

Using the *Saccharomyces* Sec59p as a probe, we were able to identify candidate dolichol kinases from all eukaryotes examined with the exception of *Giardia* (Supplemental Figure 6). As whole genome sequences were examined from two different *Giardia* isolates (WB and GS), it is unlikely that the absence of a *Giardia* dolichol kinase ortholog is due to an artifact in library construction or sequencing (Franzén et al. 2009; Morrison et al. 2007). In addition, we were unable to find in *Giardia* orthologs to *S. cerevisiae* diacylglycerol kinase (Dgk1p) (Han et al. 2008), *Arabidopsis thaliana* phytol kinase (Valentin et al. 2006), *Bacillus subtilis* undecaprenol kinase or *E. coli* diacylglycerol kinase (Lis and Kuramitsu 2003). Indeed recent work clearly demonstrates that the purified *E. coli* bacA protein (homolog of *B. subtilis* undecaprenol kinase) exhibits undecaprenyl pyrophosphate phosphatase activity but not undecaprenol kinase activity (El Ghachi et al. 2004).

Nevertheless, *Giardia* membranes have CTP-dependent dolichol kinase activity, which is increased when exogenous dolichol is added (Figure 6). *Giardia* membranes have no ATP-dependent dolichol kinase activity, as has been described for the bacterial undecaprenol kinase (Kalim and Allen 1979; Lis and Kuramitsu 2003). Similarly, *Giardia* membranes have no uridine triphosphate (UTP)- or guanosine triphosphate (GTP)-dependent dolichol kinase activity in vitro. We conclude that *Giardia* either has a deeply divergent dolichol kinase, which was not detected using the Sec59p probe, or *Giardia* has a unique dolichol kinase that does not share common ancestry with Sec59p. We cannot rule out the possibility that the *Giardia* dolichol kinase came from bacteria by LGT, but we have no evidence for this.

**Discussion**

Major conclusions include the following:

- The synthetic pathway for Dol-P is conserved in *Giardia*, even if some of the important enzymes are different from those of higher eukaryotes (e.g. *cis*-prenyltransferase) or remain unidentified (e.g. dolichol kinase). We ruled out the possibility that *Giardia* uses Dedols (as described in eubacteria) rather than dolichols, and our data suggests that the saturas of *Giardia* acts on Dedol, as described in higher eukaryotes (Kato et al. 1999).

<table>
<thead>
<tr>
<th>Dolichol kinase activity</th>
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<tr>
<td>pmol/hr/mg protein</td>
</tr>
<tr>
<td>Yeast +Dol</td>
</tr>
<tr>
<td>Yeast -Dol</td>
</tr>
<tr>
<td>Giardia +Dol</td>
</tr>
<tr>
<td>Giardia -Dol</td>
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</table>

Fig. 6. Despite the absence of an ortholog to *Saccharomyces SEC59*, *Giardia* has a CTP-dependent dolichol kinase. Membranes isolated from *Giardia* and *Saccharomyces* were incubated and radiolabeled with [*γ^32P*]CTP +/- exogenous dolichol containing 19 isoprene units. Radiolabeled alkaline-stable phospholipids, which partitioned into the organic fraction, were counted.

- The *Giardia* *cis*-prenyltransferase (GI-UPPS), which closely resembles the undecaprenyl pyrophosphate synthase of bacteria, also makes polypropenyl pyrophosphate with 11 isoprene units. Similarly, *Trypanosoma*, which makes a polypropenol with 11 isoprene units (Löw et al. 1991), has a *cis*-prenyltransferase that closely resembles the undecaprenyl pyrophosphate synthase of bacteria. These results suggest the possibility that the length of the polyprenol chain may be predicted by the ancestry of the *cis*-prenyltransferase (Clade 1 in Figure 4 are long, while Clade 2 are short). An exception to this generalization is *Plasmodium*, which has a Clade 1 *cis*-prenyltransferase but makes a polypropenol with just 11 isoprene units (D’Alexandri et al. 2006). Recent manipulations of the *Micrococcus luteus* *cis*-prenyltransferase have identified some of the active site amino acids important for determining the length of polyprenols (Kharel et al. 2006).

- GI-UPPS also makes polypropenols with 11 isoprene units in transformed *Saccharomyces rer2A, srt1A* double-deletion mutant, and there is no apparent defect in N-glycan synthesis or GPI anchor synthesis. It appears that, for the most part, dolichol kinases, Alg enzymes and Dol-P-Man synthases are not sensitive to the number of isoprene units present in their dolichols in vivo.

- Despite the absence of a *SEC59* ortholog in *Giardia*, the protozoan has a CTP-dependent dolichol kinase, as described in higher eukaryotes (Heller et al. 1992). Conversely, we were unable to identify a *cis*-prenyltransferase in *Entamoeba*.

**Materials and methods**

*Giardia* and *Saccharomyces* strains and growth conditions

Trophozoites of the first genome project WB strain of *Giardia* were grown axenically in TY1-S media supplemented with 10% serum and 1 mg/mL bile (Morrison et al. 2007). *Giardia* cells were chilled on ice for 20 min and then concentrated by centrifugation.
Saccharomyces strains used in this study, which include single deletion strains made on a BY4743 background that were obtained from Euroscarf, are listed in Table I (Brachmann et al. 1998). Yeasts were cultured in 2% (wt/vol) Bacto peptone and 1% (wt/vol) yeast extract supplemented with 2% glucose (wt/vol) (YPD, yeast peptone dextrose medium). Synthetic minimal media were made of 0.67% (wt/vol) yeast nitrogen base and 2% (wt/vol) glucose, supplemented with auxotrophic requirements. For solid media, agar (Difco, Voigt Global Distribution Inc, Lawrence, KS) was added at a 2% (wt/vol) final concentration. Sporulation of the diploid cells and tetrad dissection were performed by standard yeast genetic methods. Yeast cells were grown at 30°C and harvested at logarithmic growth phase (1 to 2 OD units/mL).

Methods to identify Giardia and Saccharomyces polyprenols
The dolichol fraction was isolated from membrane of Saccharomyces (10 mg of protein) and Giardia (40 mg of protein), as described (Grabińska et al. 2005) and subjected to analysis by liquid chromatography and mass spectrometry (LC-MS). To increase the amount of dolichol in the Giardia sample before separation of the lipids on the silica gel column, we treated prenyl phosphates with potato acid phosphatase, as described (Fujii et al. 1982).

LC/MS of lipids was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (as above). LC was operated at a flow rate of 200 μL/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 μm, 2.1 × 50 mm) was obtained from Agilent (Palo Alto, CA). The postcolumn splitter diverted ~10% of the LC flow to the electrospray ionization source of the mass spectrometer.

The LC-MS results were compared with an in vitro cis-prenyltransferase assay performed as described (Szkopinska et al. 1997). Briefly, Saccharomyces or Giardia membranes were incubated with [1-14C] IPP (60 mCi/mmol) and exogenous FPP. Gl-UPPS activity was stimulated by the presence of 0.1% Triton X-100. The length of the Dedol product was determined by reverse-phase thin layer chromatography (TLC) using standards produced in vitro by BY4741 strain of Saccharomyces and commercially available undecaprenols (American Radiolabeled Chemicals, Inc., St Louis, MO). Giardia membranes were prepared according to procedures described for preparation of Trichomonas membranes (Grabińska et al. 2008).

To make recombinant Gl-UPPS and recombinant E. coli bacA undecaprenyl pyrophosphate phosphatase in E. coli, coding sequences of each were amplified with polymerase chain reaction (PCR) and cloned into the pET30a vector (EMD Biosciences-Novagen, Madison, WI) in such a way that each protein was tagged at the N-terminus with a polyhistidine-S-tag. pET30a vectors containing the Gl-UPPS or bacA genes were each transformed into E. coli Rosetta 2 cells (Novagen). E. coli in the logarithmic growth phase were induced to express heterologous protein by incubation with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 30°C. The harvested cells were lysed by sonication, and His-tagged proteins were purified on a nickel column according to the Invitrogen protocol (Gl-UPPS) or by published methods (El Ghachi et al. 2004). Protein purity was judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blusing using a monoclonal mouse antibody against the S tag.

The cis-prenyltransferase assay was done using 10 μg of Gl-UPPS, and Dedol-PP product was dephosphorylated with undecaprenyl pyrophosphate phosphatase, as described (El Ghachi et al. 2004). Products were separated by TLC on precoated plates of silica gel 60 (Merck) using dissoxbutyl ketone/acetic acid/water (8:5:1, v/v/v) as a mobile phase.

Bioinformatic methods
The predicted proteins of Giardia of the WB strain (first genome project) and GS strain (second genome project), which have been deposited in the non-redundant (NR) database at GenBank or GiardiaDB, were searched with PSI-BLAST using cis-prenyltransferases (Rer2p and Srt1p) and dolichol kinase (Sec59p) sequences from Saccharomyces (Altschul et al. 1997; Aurrecoechea et al. 2008; Franzen et al. 2009; Heller et al. 1992; Morrison et al. 2007; Sato et al. 1999, 2001). Giardia proteins were also searched with B. subtilis undecaprenol kinase and E. coli diacylglycerol kinase (Lis and Kuramitsu 2003). Similar methods were used to search the predicted proteins of representative protists, metazoans, fungi, plants, eubacteria and archaea in the NR database at the National Center for Biotechnology Information (NCBI).

The single predicted cis-prenyltransferase of the WB strain of Giardia (GiardiaDB 50803..15256 or GenBank EDO82194) was examined for conserved domains using the CD search at the NCBI (Marchler-Bauer et al. 2005). The set of eukaryotic and prokaryotic cis-prenyltransferases was aligned using multiple sequence comparison by log-expectation (Edgar 2004). The alignment was manually refined, and gaps were removed using BioEdit. The finished alignment was used to construct the phylogenetic tree using TREE-PUZZLE, a program to reconstruct phylogenetic trees from molecular sequence data by the maximum likelihood method (Schmidt et al. 2002). Similar methods were used to draw the Sec59 tree.

Expression of Giardia cis-prenyltransferase in a Saccharomyces rer2Δ, srt1Δ double-deletion mutant
A Saccharomyces rer2Δ/RER2, srt1Δ/SRT1 double-deletion mutant (KG219) was made using a single Saccharomyces rer2Δ/RER2 deletion strain (Y23137) as a starting point. Briefly, deletion of SRT1 gene was accomplished using the plasmid pUG27 that carries the loxP-his5+-loxP gene disruption cassette (Gueldener et al. 2002). PCR primers used to target the srt1 gene were TTATAAAGAACAGGCTG-CCCTTCAACATAGGACGTTCCTCTGACATACA- CAGCTGAAAGCTTGCTCTGACGC (sense) and TTCAGATGTTCCTTGGCCCTCTCTTGGGCTTTTC- TAGTTTTGACATTATTACGATAGGCCACATGTTG- GATCTG (antisense). The pUG27-srt1 knock-out construct was transformed into Y23137 yeast cells. Transformants able to grow on medium lacking histidine and containing G418 were isolated, and the correct insertion of the deletion cassette
was verified by PCR. The double heterozygous mutant rer2::kanMX4/rer2, srt1::his3MX6loxP/SRT1 was called KG219.

The coding sequence of putative *Giardia cis*-prenyltransferase, which was fused to the EK11 ER-retention signal, was amplified from WB strain genomic DNA using two custom primers. The sense primer, which contained a HindIII restriction enzyme site in bold, was AAAAAAGCTTATGATCCCCATG- CATGTGGC. The antisense sequence, which contained a SalI restriction site in bold and encoded EK11N in italic, was TTTGGCAGCTACAATTCAACTTTTTTGTATGGTCATGATGGGEE. The PCR product was cloned into the pGEM-T Easy vector and sequenced (Promega, Madison, WI).

To express *Giardia cis*-prenyltransferase (GI-UPPS) in the *Saccharomyces* cells, a NotI-surrendered insert was sub-cloned into the pNEV-N plasmid under the control of the *PM11* promoter and terminator (Sauer and Stolz 1994). The pNEV–GI-UPPS plasmid was transformed into the KG219 yeast strain. Yeasts were sporulated, and colonies were selected that were resistant to G418 and able to grow on the medium lacking histidine. These yeasts, which express the *Giardia cis*-prenyltransferase in a *Saccharomyces rer2*, *srt1Δ* double-deletion mutant, were labeled KG119. A single deletion mutant rer2Δ that expressed GI-UPPS was called KG120.

KG119 and its wild-type counterpart BY4741 were characterized in six ways. 1) Polyprenols were extracted and characterized by mass spectroscopy, as described above for *Giardia*. 2) Membranes were isolated and incubated with radiolabeled IPP and exogenous FPP, and radiolabeled Dedols were demonstrated by reverse-phase TLC, as described above. 3) The N-glycosylation status of carboxypeptidase (CPY) was determined by western blotting of *Saccharomyces* glycoproteins with antibodies to CPY (Molecular Probes, Eugene, OR) before or after peptide-N-glycanase treatment. A negative control was a *Saccharomyces* alg5Δ mutant (Heesen et al. 1994). 4) Maturation of the GPI anchors present on the *Saccharomyces* Gas1p (Gatti et al. 1994) was determined by western blotting of *Saccharomyces* glycoproteins with antibodies to Gas1p (kind gift of Laura Popolo). A negative control was a *Saccharomyces* gpl1 thermosensitive mutant (Leidich et al. 1994). 5) Expression of SRT1 mRNAs was measured in wild type versus *Saccharomyces rer2Δ* with or without the exogenous GI-UPPS. 6) Cell wall stress was indirectly determined by measuring chitin levels (Popolo et al. 1997). Chitin content was measured by an assay adapted for microtiter plates, as described (Grabińska et al. 2007).

**Dolichol kinase assay**

CTP, GTP and UTP were synthesized enzymatically from cytidine diphosphate, guanosine diphosphate or uridine diphosphate and [γ-32P]ATP with nucleoside-5′-diphosphate kinase, as described (Han et al. 2008). The dolichol kinase assay was performed, as described (Heller et al. 1992). Membrane fractions (200 μg) were incubated in a total volume of 100 μL containing 0.05 M Tris–HCl (pH 7.5), 10 mM UTP, 100 mM CaCl2, 0.02–0.1 μCi [γ-32P]UTP in 0.1% Triton X-100 and 2 μg of dolichol mixture for 30 min at room temperature. Alternatively, [γ-32P]ATP or [γ-32P]GTP was used instead of [γ-32P]CTP. When [γ-32P]UTP substituted for [γ-32P]CTP, then 10 mM ATP was used instead of 10 mM UTP. Reactions were terminated by the addition of 750 μL of 1 M KOH in methanol, and alkali-labile lipids were hydrolyzed by incubation at 37°C for 30 min. This step is required to hydrolyze phosphatidic acid. The lipids were extracted by the Folch method, and the [32P] incorporation into Dol-P was determined by scintillation counting.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest statement**

None declared.

**Abbreviations**

ATP adenosine triphosphate; CPY, carboxypeptidase Y; CTP cytidine triphosphate; Dedol, dehydrodolichol; Dedol-PP, dehy-drodiolylch pyrophosphate; Dol-P, dolichol phosphate; Dol-P-Man, dolichol phosphate mannoside; FOA, 5-fluoroorotic acid; FPP, farnesyl pyrophosphate; Gas1p, glucanosyltransferase encoded by GAS1; GI-UPPS, *Giardia lamblia* undecaprenol pyrophosphate synthase; GPI, glycosylphosphatidylinositol; GTP guanosine triphosphate; IPP, isopentenyl pyrophosphate; LC-MS, liquid chromatography and mass spectrometry; LGT, lateral gene transfer; NCBI National Center for Biotechnology Information; N-glycan, Asn-linked-glycan; NR non-redundant; PCR polymerase chain reaction; TLC, thin layer chromatography; UTP uridine triphosphate; YPD, yeast peptone dextrose medium.

**References**


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thesis, is essential for carrier lipid formation in bacterial cell wall synthesis.

Escherichia coli

Saccharomyces cerevisiae

Streptococcus

Giardia intestinalis

Plasmodium falciparum

Mycobacterium tuberculosis

Saccharomyces cerevisiae

Escherichia coli

Saccharomyces cerevisiae

Giardia lamblia

Giardia

Saccharomyces cerevisiae

Saccharomyces cerevisiae

Saccharomyces cerevisiae

Saccharomyces cerevisiae

Saccharomyces cerevisiae

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