

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 polyprenol 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 *Saccharomyces cerevisiae* SEC59 gene product), we wondered how *Giardia* synthesizes dolichol phosphate (Dol-P), which is used to make *N*-glycans and glycosylphosphatidylinositol (GPI) anchors. Here we show that cultured *Giardia* makes an unsaturated polyprenyl pyrophosphate (dehydrodolichol), which contains 11 and 12 isoprene units and is reduced to dolichol. The *Giardia cis*-prenyltransferase that we have named GI-Upps because the enzyme primarily synthesizes undecaprenol pyrophosphate is phylogenetically related to those of bacteria and *Trypanosoma* rather than to those of other protists, metazoans and fungi. In transformed *Saccharomyces*, the *Giardia cis*-prenyltransferase also makes a polyprenol containing 11 and 12 isoprene units and supports normal growth, *N*-glycosylation and GPI anchor synthesis of a *rer2Δ*, *srt1Δ* double-deletion mutant. Finally, despite the absence of an ortholog to SEC59, *Giardia* has cytidine triphosphate-dependent dolichol kinase activity. These results suggest that the synthetic pathway for Dol-P is conserved in *Giardia*, even if some of the important enzymes are different from those of higher eukaryotes or remain unidentified.

Keywords: *cis*-prenyltransferase/dolichol/*Giardia*/
recombinant expression/*Saccharomyces*

Introduction

Giardia lamblia, which is spread by the fecal–oral route, is an important parasitic cause of diarrhea in developing and developed countries (Adam 2001; Savioli et al. 2006). *Giardia* is

remarkable for the presence of two similar nuclei and a genome that contains a large number of genes obtained from bacteria by lateral gene transfer (LGT) (Andersson et al. 2003; Franzén et al. 2009; Morrison et al. 2007).

In this report, we describe *Giardia* enzymes that synthesize dolichol phosphate (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) (Helenius 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,

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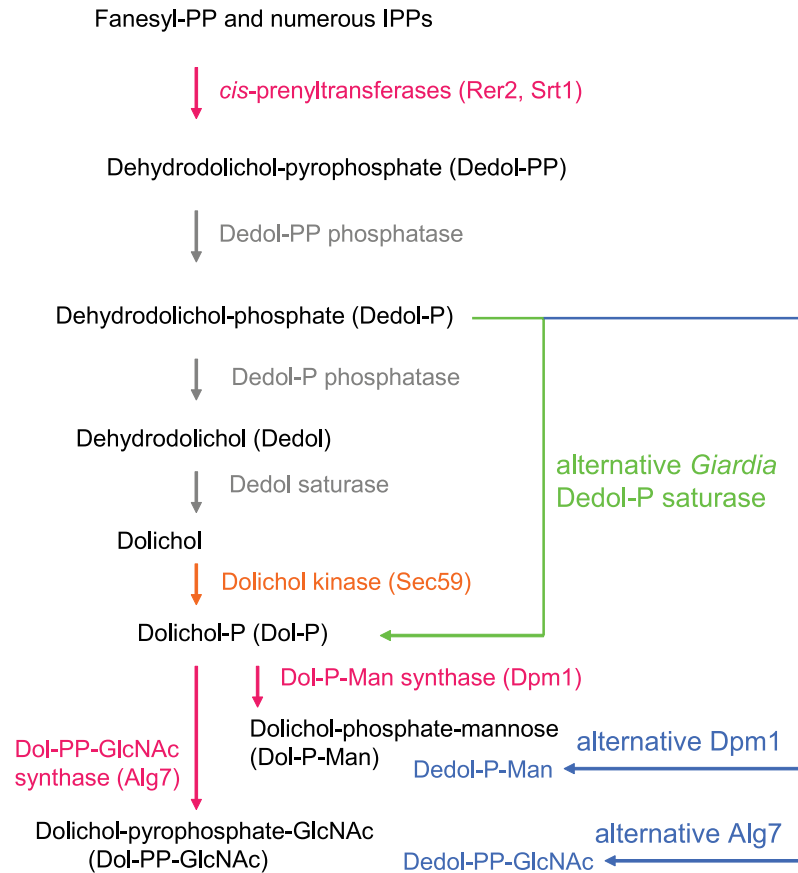


Fig. 1. Mapping of predicted *Giardia* enzymes onto the eukaryotic synthetic pathway for dolichol phosphate (Dol-P). Orthologs to *Saccharomyces* enzymes, which are present in *Giardia* (e.g. Rer2/Srt1, Alg7 and Dpm1) are marked in red. *Saccharomyces* Sec59, which appears to be absent in *Giardia*, 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).

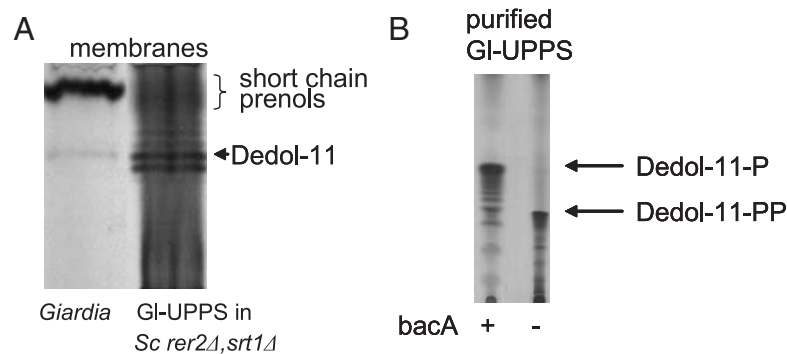


Fig. 2. Thin layer chromatography (TLC) shows the *Giardia* undecaprenyl pyrophosphate synthase (GI-UPPS), which closely resembles the bacterial UPPS (Supplement Figure 1 and see Figure 4 below) that makes a dominant polyprenol pyrophosphate with 11 isoprene units (Dedol-11). **(A)** In the left lane, *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 polyprenol 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.

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)?

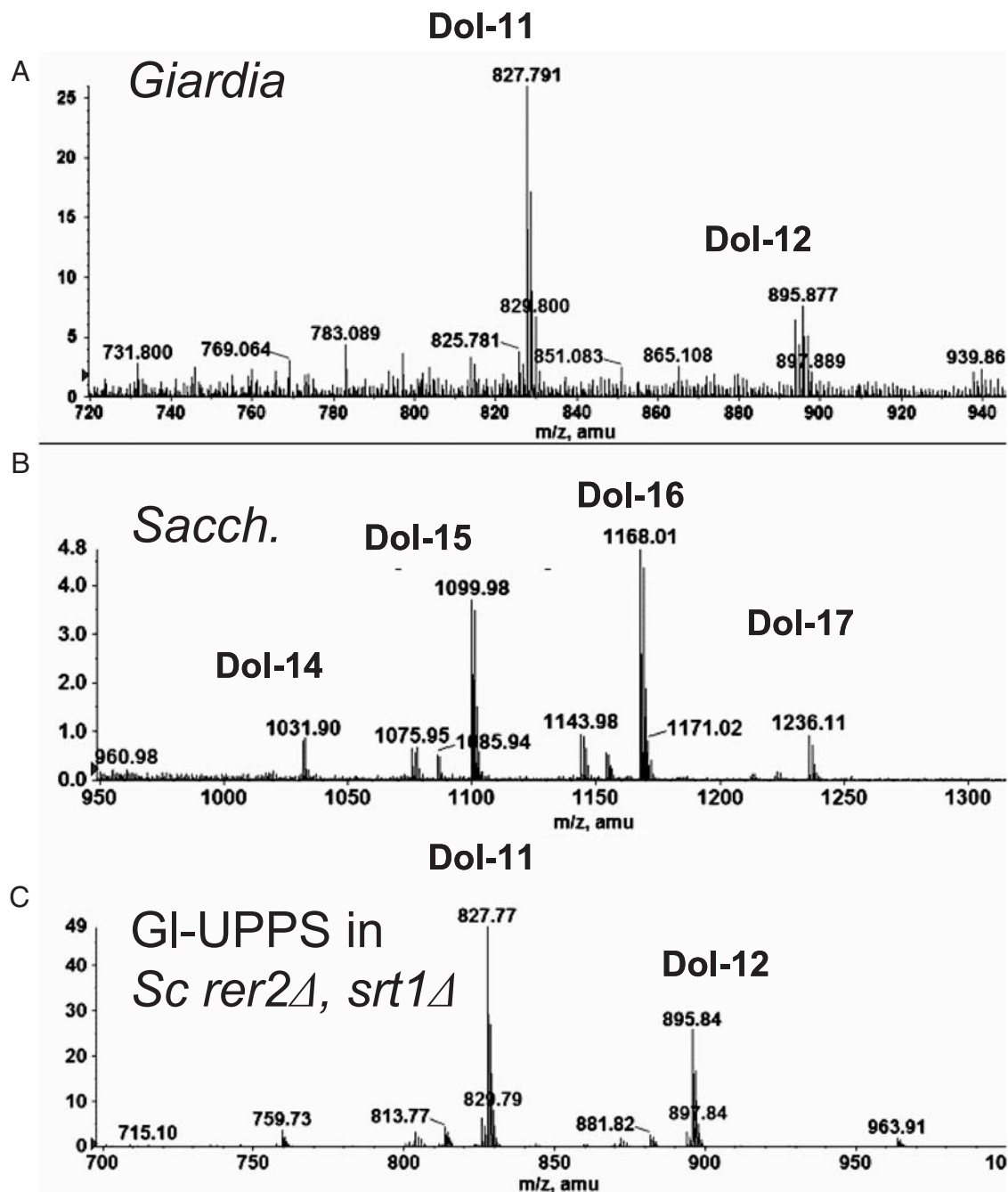


Fig. 3. Mass spectrometry of the products of the *Giardia cis*-prenyltransferase (GI-UPPS) reveals dolichols containing 11 and 12 isoprene units. (A) Dolichols extracted from *Giardia* trophozoites growing in axenic culture (without bacteria) include Dol-11 and Dol-12. The absence of phosphate and pyrophosphate groups on the polyprenols implies the existence of endogenous *Giardia* Dedol-PP pyrophosphatases and Dedol-P phosphatases. (B) Dolichols isolated from wild-type *Saccharomyces* (BY4741) containing intact *RER2* and *SRT1* genes contain 14 to 17 isoprene units. (C) Transformed *Saccharomyces rer2Δ, srt1Δ* double-mutant, which is expressing GI-UPPS, makes Dol-11 and Dol-12. While the *Saccharomyces rer2Δ, srt1Δ* double-mutant is dependent upon expression of GI-UPPS for growth (Figure 5), these cells do not show defects in *N*-glycan or GPI anchor synthesis, using CPY and Gas1p, respectively, as reporters (Supplemental Figures 2 and 3, respectively).

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).

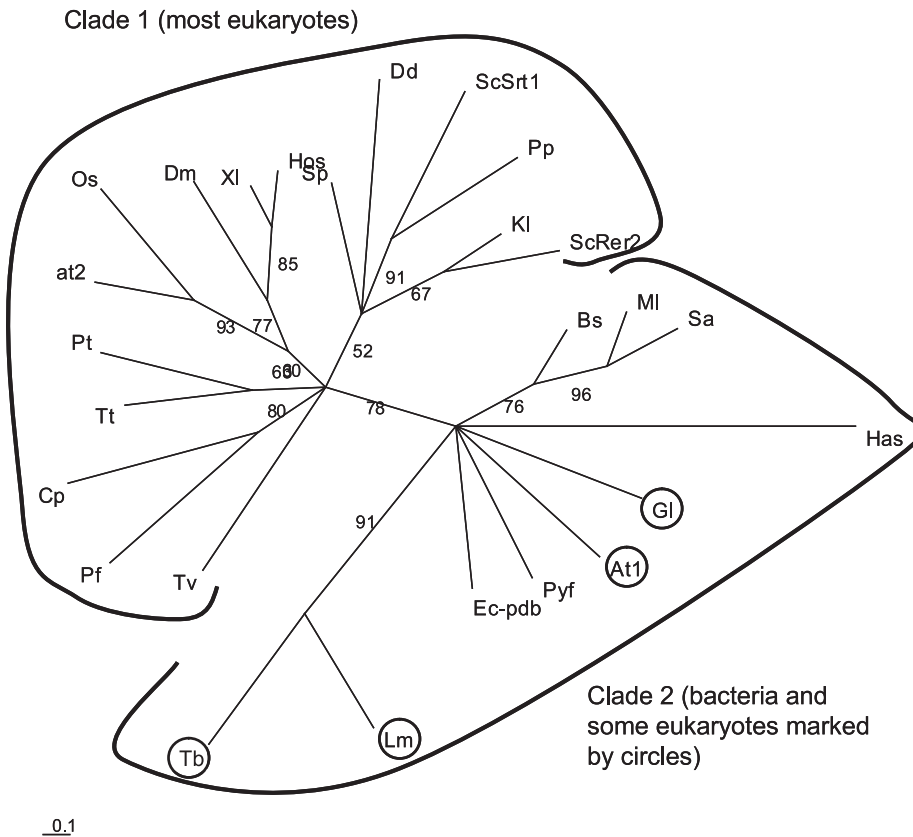


Fig. 4. The *Giardia* undecaprenyl pyrophosphate synthase (GI-UPPS) resembles *cis*-prenyltransferases of eubacteria, archaea, *Trypanosoma* and plant chloroplast. In the phylogenetic tree, which was constructed by maximum likelihood methods, the branch lengths are proportionate to differences between sequences, while the nodes indicate bootstrap support. Nodes with less than 50% bootstrap support were collapsed. Representative protists include *Giardia* (GI), *Cryptosporidium parvum* (Cp), *Dictyostelium discoideum* (Dd), *Leishmania major* (Lm), *Paramecium tetraurelia* (Pt), *Plasmodium falciparum* (Pf), *Tetrahymena thermophila* (Tt), *Trichomonas vaginalis* (Tv) and *Trypanosoma brucei* (Tb). Representative metazoans include *Drosophila melanogaster* (Dm), *Homo sapiens* (Hos) and *Xenopus laevis* (Xl). Representative fungi include *Kluyveromyces lactis* (Kl), *Pichia pastoris* (Pp), *Saccharomyces cerevisiae* (ScRer2 and ScSrt1) and *Schizosaccharomyces pombe* (Sp). Also included are chloroplast (At1) and cytosolic (At2) *cis*-prenyltransferases of *Arabidopsis thaliana*, as well as that of *Oryza sativa* (Os). Representative eubacteria include *Escherichia coli* (Ec-PDB) and *Micrococcus luteus* (MI), each of which has been crystallized (Fujihashi et al. 2001; Guo et al. 2005), as well as *Bacillus subtilis* (Bs) and *Staphylococcus aureus* (Sa). Representative archaea include *Pyrococcus furiosus* (Pyf) and *Halobacterium* sp. (Has).

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 Dol-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 GI-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-

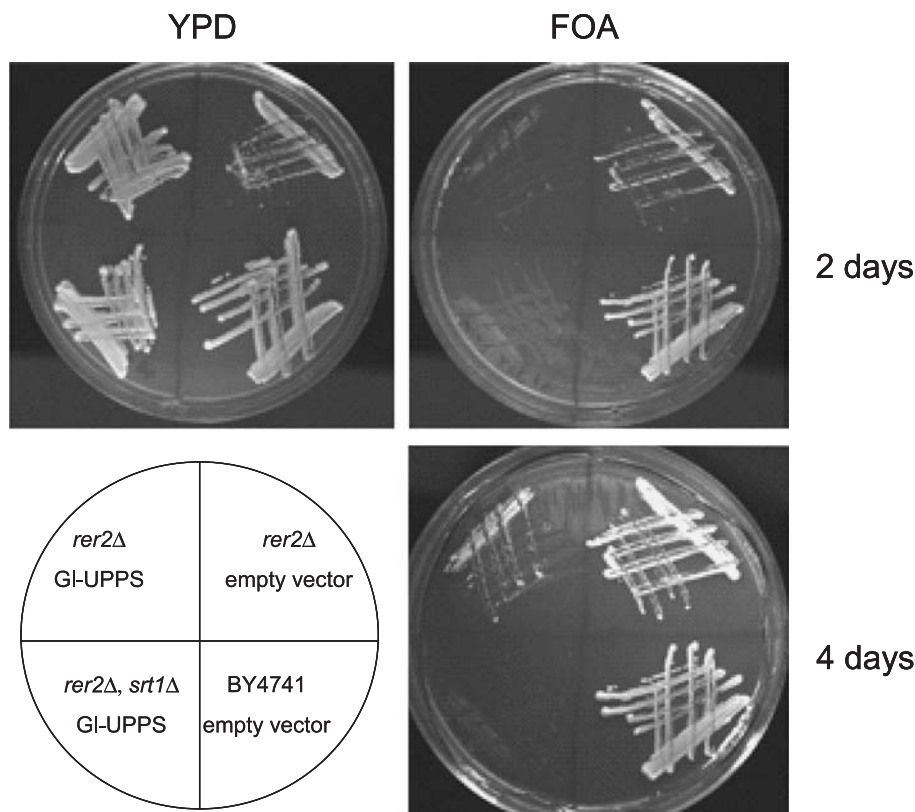


Fig. 5. Functional complementation of *Saccharomyces rer2Δ, srt1Δ* double-deletion mutant by the *Giardia cis*-prenyltransferase (GI-Upps). Wild-type yeast (BY4741), the *rer2Δ* deletion strain, the *rer2Δ* strain expressing GI-Upps or the *rer2Δ, srt1Δ* double-deletion strain expressing GI-Upps were 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 GI-Upps expressing plasmid.

Table I. Yeast strains

Source	Strain name	Genotype/description
Euroscarf	BY4741	<i>Mat a his3Δ1 leu2Δ1 met15Δ01 ura3Δ1</i>
Euroscarf	BY4743	<i>MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0</i>
Euroscarf	Y23137	<i>MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 rer2::kanMX4/RER2</i>
This study	KG219	<i>MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 LYS2/LYS2 ura3Δ0/ura3Δ0 rer2::kanMX4/RER2 srt1::his3MX6loxP/SRT1</i>
This study	KG119	As for BY474, <i>rer2::kanMX6 srt1::his3MX6loxP/pNEV-GI-Upps</i>
This study	KG120	As for BY474, <i>rer2::kanMX6 pNEV-GI-Upps</i>
Leidich et al. 1994	<i>gpi1</i>	<i>MAT α gpi1 ura3-52 his4-917 leu2-3</i>
Euroscarf	<i>alg5</i>	As for BY474, <i>alg5::kanMX6</i>

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 (GI-Upps) complements a *Saccharomyces rer2Δ, srt1Δ* double-deletion mutant (Figure 5

and Table I). The necessity of the GI-Upps is shown by the absence of growth of *rer2Δ, srt1Δ* double-deletion mutant when the plasmid containing the GI-Upps gene is lost from yeast after treatment with 1% (w/v) 5-fluoroorotic acid (FOA). GI-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 glucanoyltransferase encoded by GAS1 (Gas1p) as the reporter) (Supplemental Figure 3). While there is induction of *SRT1* mRNA in the *Saccharomyces rer2Δ* mutant, there is

no induction of *SRT1* mRNA when the *Saccharomyces rer2Δ* mutant is complemented by the GI-UPPS gene (Supplemental Figure 4). There is, however, a mild increase in the synthesis of chitin (consistent with mild cell wall stress) in *Saccharomyces rer2Δ*, *srt1Δ* double-deletion mutant complemented with GI-UPPS (Supplemental Figure 5).

When the GI-UPPS with a histidine tag was expressed in *E. coli*, the recombinant protein, which was purified using a nickel column, made prenol-11-PP when incubated with radiolabeled IPP and exogenous FPP (Figure 2). This result suggests that the activity of the GI-UPPS is not dependent upon accessory proteins.

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 (Kalin 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 saturase of *Giardia* acts on Dedol, as described in higher eukaryotes (Kato et al. 1999).

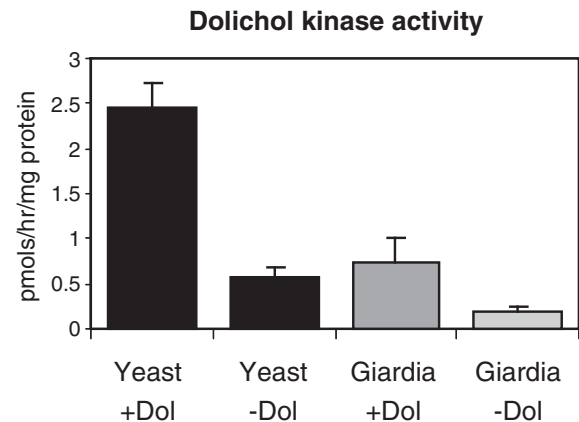


Fig. 6. Despite the absence of an ortholog to *Saccharomyces* SEC59, *Giardia* has a CTP-dependent dolichol kinase. Membranes isolated from *Giardia* and *Saccharomyces* were incubated and radiolabeled with [γ - 32 P]CTP +/- exogenous dolichol containing 19 isoprene units. Radiolabeled alkaline-stable 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 polyprenyl pyrophosphate with 11 isoprene units. Similarly, *Trypanosoma*, which makes a polyprenol 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 polyprenol 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 polyprenols with 11 isoprene units in transformed *Saccharomyces rer2Δ*, *srt1Δ* 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 protist 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 TYI-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 μ L/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 μ m, 2.1 \times 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 [$1\text{-}^{14}\text{C}$] 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 ex-

press 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 blotting using a monoclonal mouse antibody against the S tag.

The *cis*-prenyltransferase assay was done using 10 μ g 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 pre-coated plates of silica gel 60 (Merck) using diisobutyl 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) data 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; Aurecochea et al. 2008; Franzén 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 rer2 Δ , srt1 Δ* double-deletion mutant

A *Saccharomyces rer2 Δ /RER2, srt1 Δ /SRT1* double-deletion mutant (KG219) was made using a single *Saccharomyces rer2 Δ /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 (Guedener et al. 2002). PCR primers used to target the *srt1* gene were TTATAAAGAACAGGCTG-CCTTTCAAACATAGGACGTTTCTGTTGACCATA-CAGCTGAAGCTTCGTCTTCGTACGC (sense) and TTCAGAATGTTCTTTGCCCTCTCTTGGGCTTTC-TAGTTTTGCACTTTTACGCATAGGCCACTAGTG-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 EKKL 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 **AAAAAGCTTATGATCCCCATG-CATGTGGC**. The antisense sequence, which contained a Sall restriction site in bold and encoded EKKLN in italic, was **TTTGTCTGACTCAATTCAACTTTTTTCGTGTCATGGTTC-GATAGGG**. 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 subcloned into the pNEV-N plasmid under the control of the *PMA1* 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 rer2Δ*, *srt1Δ* double-deletion mutant, were labeled KG119. A single deletion mutant *rer2Δ* that expressed GL-UPPS was called KG120.

KG119 and its wild-type counterpart BY4741 were characterized in six ways. 1) Polyphenols 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*-glycanaseF 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 gpi1* 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 μ g) were incubated in a total volume of 100 μ L containing 0.05 M Tris-HCl (pH 7.5), 10 mM UTP, 100 mM CaCl₂, 0.02–0.1 μ Ci [γ -³²P]CTP in 0.1% Triton X-100 and 2 μ g 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 μ L

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, dehydrololichol; Dedol-PP, dehydrololichyl pyrophosphate; Dol-P, dolichol phosphate; Dol-P-Man, dolichol phosphate mannose; 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.

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