Dolichyl-Phosphate-Glucose Is Used To Make O-Glycans on Glycoproteins of Trichomonas vaginalis

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Received 15 February 2008/Accepted 2 May 2008

Trichomonas vaginalis, the protist that causes vaginal itching, has a huge genome with numerous gene duplications. Recently we found that Trichomonas has numerous genes encoding putative dolichyl-phosphate-glucone (Dol-P-Glc) synthases (encoded by ALG5 genes) despite the fact that Trichomonas lacks the glycosyltransferases (encoded by ALG6, ALG8, and ALG10 genes) that use Dol-P-Glc to glucosylate dolichyl-PP-linked glycans. In addition, Trichomonas does not have a canonical DPM1 gene, encoding a dolichyl-P-mannose (Dol-P-Man) synthase. Here we show Trichomonas membranes have roughly 300 times the Dol-P-Glc synthase activity of Saccharomyces cerevisiae membranes and about one-fifth the Dol-P-Man synthase activity of Saccharomyces cerevisiae membranes. Endogenous Dol-P-P-heptoses of Trichomonas are relatively abundant and contain 16 isoprene units. Five paralogous Trichomonas ALG5 gene products have Dol-P-Glc synthase activity when expressed as recombinant proteins, and these Trichomonas Alg5s correct a carboxypeptidase N glycosylation defect in a Saccharomyces al5 mutant in vivo. A recombinant Trichomonas Dpm1, which is deeply divergent in its sequence, has Dol-P-Man synthase activity. When radiolabeled Dol-P-Glc is incubated with Trichomonas membranes, Glc is incorporated into reducing and nonreducing sugars of O-glycans of endogenous glycoproteins. To our knowledge, this is the first demonstration of Dol-P-Glc as a sugar donor for O-glycans on glycoproteins.

UDP-glucose:dolichyl-phosphate β-D-glucosyltransferase (Dol-P-Glc synthase; EC 2.4.1.117), which is encoded by the ALG5 gene in Saccharomyces cerevisiae, converts cytoplasmic UDP-Glc and endoplasmic reticulum (ER) membrane-bound Dol-P to Dol-P-Glc (9, 11). On the luminal face of the ER, Dol-P-Glc serves as the sugar donor for transfer of three glucose residues to the Man9GlcNAc2-PP-dolichol precursor to Asn-linked glycans (N-glycans) (10). The glucosyltransferases, which are called Alg6, Alg8, and Alg10 in Saccharomyces, make the 14-sugar N-glycan precursor (Glc3Man9GlcNAc2-PP-dolichol), which is transferred by the oligosaccharyltransferase to Asn residues on nascent glycoproteins in the ER lumen (15).

GDP-mannose:dolichyl-phosphate β-D-mannosyltransferase (Dol-P-Man synthase; EC 2.4.1.83), which is encoded by DPM1 in Saccharomyces (a paralog of ALG5), converts cytoplasmic GDP-mannose and Dol-P to Dol-P-Man (22, 23). On the luminal face of the ER, Dol-P-Man serves as a mannose donor for three separate groups of enzymes. (i) Mannosyltransferases, encoded by ALG3, ALG9, and ALG12 genes in Saccharomyces, use Dol-P-Man to convert the N-glycan precursor Man9GlcNAc2-PP-dolichol to Man9GlcNAc2-PP-dolichol (which is subsequently glucosylated to Glc3Man9GlcNAc2-PP-dolichol) (10).

(ii) Mannosyltransferases, encoded by GPI genes in Saccharomyces and PIG genes in mammals, use Dol-P-Man to make glycosylphosphatidylinositol (GPI) anchors for GPI-linked membrane proteins (24). (iii) Mannosyltransferases, encoded by PMT genes in Saccharomyces and POMT genes in mammals, use Dol-P-Man to add single O-linked mannose residues to serine/threonine residues of glycoproteins in the ER lumen (20, 35).

Trichomonas vaginalis is the protist that causes vaginitis and increases the risk of transmission of human immunodeficiency virus (28, 34). The 150-Mb genome of Trichomonas, which predicts 60,000 proteins, is by far the largest of any protist (4). We are interested in protein glycosylation in Trichomonas for the following reasons. First, Trichomonas is missing glycosyltransferases, which use Dol-P-Man and Dol-P-Glc to add mannose and glucose residues to N-glycan precursors in the lumen of the ER (25). Therefore, Trichomonas makes a seven-sugar N-glycan precursor (Man9GlcNAc2-PP-dolichol), which is transferred by the oligosaccharyltransferase to Asn on nascent peptides.

Second, Trichomonas has multiple putative ALG5 genes, which likely synthesize Dol-P-Glc, even though there is no obvious use for Dol-P-Glc by the protist (25). Third, Trichomonas is the only eukaryote we have examined that does not have a canonical Dol-P-Man synthase gene (DPM1) (25). Fourth, Trichomonas is missing the enzymatic machinery for making GPI anchors, an important posttranslational modification in all other eukaryotes examined to date (4, 24). Fifth, Trichomonas makes a unique lipophosphoglycan (LPG), which is antigenic and is involved in pathogenesis (3, 8, 29).
Several specific questions were asked here. Does *Trichomonas* synthesize Dol-P-Glc and Dol-P-Man? Which candidate *ALG5* genes of *Trichomonas* synthesize Dol-P-Glc and/or Dol-P-Man when expressed in a heterologous system? What is the in vitro product when Dol-P-Glc is incubated with *Trichomonas* membranes?

**MATERIALS AND METHODS**

**Materials.** UDP-glucose, GDP-mannose, and [1-14C]sphingosine pyrophosphate (60 mCi/mmol) were purchased from Sigma-Aldrich. UDP-[6-3H]glucose (60 mCi/mmol), UDP-[1-14C]glucose (300 mCi/mmol), GDP-[2-3H]mannose (20 Ci/mmol), GDP-[U-14C]mannose (298.1 mCi/mmol), and sodium [3H]sphingosine (2Ci/mmol) were purchased from American Radiolabeled Chemicals. Antibodies used in Western blots included mouse anti-S-tag (Novagen), mouse anti-carboxyvas deferens (Tissue Culture) and horseradish peroxidase-conjugated secondary antibodies (Promega). Peptide-N-glycosidase F (PNGase F) was from New England Biolabs. All other chemicals were of the highest quality available.

Dol-P-Glc and Dol-P-Man synthase activities of *Trichomonas* membranes. The U1 isolate of *Trichomonas vaginalis* (8) was grown in liquid TYM medium supplemented with 10% heat-inactivated bovine serum (Invitrogen). Cultured *Trichomonas* cells were chilled, washed three times with phosphate-buffered saline, resuspended in a buffer composed of 50 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol, and 0.15 mM MgCl₂, and sonicated on ice for 30 seconds. Large debris was removed by low-speed centrifugation, and *Trichomonas* membranes were obtained by centrifugation for 1 h at 100,000 x g. The membranes were washed fresh or were stored at −80°C in the same buffer plus 3.5 mM MgCl₂. As a control, fresh and frozen membranes were prepared from the BY4741 strain of *Saccharomyces cerevisiae* or its derivative (Euroscarf), which was grown in synthetic complete medium and disrupted with glass beads. In our experience, freezing decreases the enzyme activities of *Trichomonas* and Saccharomyces membranes by 10 to 15%.

The Dol-P-Glc synthase activity of 100 µg of *Trichomonas* or Saccharomyces membranes was determined in 50-µl reaction mixtures containing 10 µg of exogenous Dol-P as a sugar acceptor, 0.1% NP-40, 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.4), and radiolabeled UDP-Glc. Conditions for these assays were determined by varying the concentration of *Trichomonas* membranes and by varying the pH with recombinant *Trichomonas* enzymes (see Fig. S1 in the supplemental material). For Dol-P-Man synthase activities, membranes were incubated with radiolabeled GDP-Man (17). After 5 to 15 min at 30°C, reactions were stopped by addition of 4 ml of chloroform-methanol (3/2, vol/vol). Organic phases containing radiolabeled Dol-P-Glc or Dol-P-Man were washed once with 1 ml of 4 mM MgCl₂ and twice with 0.8 ml of chloroform-methanol-4 mM MgCl₂ (3/48/47). The radioactivity incorporated into the lipid fraction was measured by liquid scintillation counting. The synthetic activity of the purified lipid was confirmed by comigration with a *Saccharomyces* Dol-P-Glc standard by thin-layer chromatography (TLC) on silica gel plates developed in chloroform-methanol-water (65/25/4). The sensitivity of the product to mild acid treatment (10 min of hydrolysis in 40 mM trifluoroacetic acid at 90°C) was tested, and the identity of the released sugar as Glc or Man was confirmed by comigration with an internal standard by high-ph pH anion-exchange chromatography as described previously (33).

**Purification and characterization by mass spectrometry of endogenous *Trichomonas* Dol-P-hexose.** Membranes from *Trichomonas* grown in a 2-liter culture were extracted with chloroform-methanol (3/2). The organic phase was washed once with 1/5 volume of 4 mM MgCl₂ and twice with chloroform-methanol-4 mM MgCl₂ (3/48/47). Dried lipids were dissolved in 5 ml of chloroform-methanol-water (2/3/1) and separated on a 5-ml DEAE-cellulose column equilibrated in the acetate form, as described previously (14).

Liquid chromatography (LC)/mass spectrometry of lipids was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps, and a SPD-10A system controller) coupled to a QSTAR XL quadrupole time of flight tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray source. LC was operated at a flow rate of 200 µl per 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 B consisted of methanol/acetonitrile-acetic acid 1-mM ammonium acetate (60/40/20). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 µm, 2.1 by 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. Negative-ion mass spectra were acquired with an electrospray voltage of −4.500 V.

The LC/mass spectrometry results were compared with those of an in vitro cis-prenyltransferase assay (32), in which *Trichomonas* membranes were incubated with radiolabeled isopentenyl pyrophosphate and exogenous farnesyl pyrophosphate in the presence of 0.1% Triton X-100. The length of the dehydromannitol product was determined by reverse-phase TLC using dehydromannitol produced in vitro by Saccharomyces as a standard.

In silico identification of Dol-P-Glc and Dol-P-Man synthases from *Trichomonas*. *Saccharomyces cerevisiae* Alg5p (Dol-P-Glc synthase) and Dpm1p (Dol-P-Man synthase) were used to search predicted proteins of *Trichomonas vaginalis* using BLASTP or TBLASTN in the databases managed by The J. Craig Venter Institute (http://www.tigr.org/db/ebi2/7g (14)). This resulted in five putative *Trichomonas* *ALG5* genes (TvALG5A to TvALG5E), as well as weak hit (TvALG5G). TvALG5G was in turn used to identify the *TvDPM1* gene, which was present in two overlapping protein predictions. The corrected *TvDPM1* gene sequence was determined by sequencing a *TvDPM1* PCR product made with primers to the 5’ and 3’ ends of the coding sequence. Comparison of PCR products obtained with genomic DNA and cDNA as a template revealed that none of the *Trichomonas* *Alg5p* or *Dpm1p* genes contain an intron.

Transmembrane helices in the *Trichomonas* Dol-P-Glc and Dol-P-Man synthases were predicted by using the Phobius combined transmembrane topology and signal peptide predictor (13). Alignments of protein sequences were made using Clustal W (http://www.ebi.ac.uk/clustalw), and multiple alignments and trimming of the alignments were performed with Jalview (7, 18). Phylogenetic trees were constructed from the positional variation with maximum likelihood by using quartet puzzling (12, 27).

Cloning and recombinant expression of *Trichomonas* Dol-P-Glc and Dol-P-Man synthase genes. The coding sequences of putative *Trichomonas* glycosyltransferase genes and *Saccharomyces* *ALG5* and *DPM1* genes were amplified using genomic DNA as a template and a pair of primers listed in Table S1 in the supplemental material. PCR products were cloned into the pGEM-T Easy vector and sequenced (Promega).

To express *Trichomonas* genes in the Saccharomyces cells, NotI-situated inserts were subcloned into the pNEV-N plasmid under the control of the PMA1 promoter and terminator (see Table S1 in the supplemental material) (26). pNEV constructs containing *Trichomonas* *ALG5* genes, as well as the *Saccharomyces* *ALG5* gene, were transformed into the BY4741 derivative alg5Δ yeast strain (Euroscarf). As a second positive control, wild-type BY4741 cells transformed with empty vector pNEV-N were also tested. Membrane fractions were isolated from transformed yeast and tested for Dol-P-Glc synthase activity as described above.

To examine the functionality of *TvAlg5* proteins in vivo, we tested the N glycosylation status of carboxypeptidase Y in *Saccharomyces* strains transformed with plasmids carrying *Trichomonas ALG5* genes in combination with wild-type and *Saccharomyces* mutant *Saccharomyces* strains (9). Yeast proteins were obtained by alkaline lysis and resolved on sodium dodecyl sulfate (SDS)-NuPAGE Novex Bis-Tris (4–12%) gels under reducing conditions. Gels were transferred to polyvinylidine difluoride membranes, which were blocked in 5% milk and probed with antibodies against *Saccharomyces* carboxypeptidase Y.

To express *Trichomonas* genes in *Escherichia coli*, BamHINotI fragments were cloned into the pET30a vector (Novagen) in such a way that the protein was tagged at the N terminus with a histidine-S-tag fusion, pET30a vectors containing the ScDPM1, *TvALG5E*, or *TvDPM1* gene were each transformed into *Escherichia coli* Rosetta 2 cells (Novagen). E. coli cells in the logarithmic growth phase were induced to express heterologous protein by incubation with 1 mM IPTG (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. Protein purity was judged by SDS-polyacrylamide gel electrophoresis and Western blotting using monoclonal mouse antibody against the S tag. The amount of the purified protein was estimated using the *S*Tag rapid assay kit (Novagen), and 0.1 pmol of purified enzyme was added to the Dol-P-Glc or Dol-P-Man synthase assay mixtures.

Real-time PCR. Total RNA was isolated from mid-log-phase *Trichomonas* cells using the RNeasy kit (Qiagen), and RNA was treated with DNA-free reagent (Ambion) in accordance with the manufacturer’s instructions. Reverse transcription of RNA was carried out with the RETROscript kit (Ambion) using oligo(dT)₁₆. Levels of specific mRNA species were measured by real-time PCR using the Sybr green method on a Bio-Rad Big plate format, and a Stratagene MX4000 cycle. Reaction mixtures contained 12.5 µl 2X Brilliant Sybr green quantitative PCR master mix (Stratagene), primers (100 nM), and a template in a total volume of 25 µl. Master mix was made with reverse-transcribed RNA to minimize the sampling error. The thermal profile for amplification was 95°C for 10
min, followed by 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. Primers for real-time PCR were designed (see Table S1 in the supplemental material) using OligoPerfect software (Invitrogen). Gel electrophoresis was carried out on representative samples to confirm product size. Amplification plots were analyzed using MX400 software, version 4.20 (Stratagene), to determine the relative quantity of mRNA species using a *Trichomonas* actin gene as the calibrator.

In *in vitro* glucosylation of *Trichomonas* proteins using radiolabeled Dol-P-Glc. Radiolabeled dolichyl-[14C]glucose was synthesized by incubating commercially available UDP-[14C]Glc and Dol-P with recombinant *TvAlg5E*, which was purified from *E. coli* as described above. Radiolabeled Dol-P-Glc was extracted into chloroform, and its identity was confirmed by its mobility in TLC and by its sensitivity to mild acid hydrolysis. The reaction mixture, which was incubated at 30°C for 60 min in a volume of 250 μl, contained 0.5% octyl-o-thioglucoside, 10 mM MnCl₂, 50 mM Tris-HCl, pH 7.4, 20,000 cpm Dol-P-[14C]Glc, and 500 μg of *Trichomonas* membranes. Control reaction mixtures were incubated for 0 min, incubated at 0°C, incubated at pH 3 or pH 10, or incubated in the presence of EDTA (see Fig. S2 in the supplemental material). An additional control used membranes heated to 100°C for 2 min to denature the Dol-P-Glc-dependent glucosyltransferase.

Reactions were stopped with 4 ml of chloroform-methanol (3/2), and the combined pellets from 20 reactions were delipiddated four times with 10 ml of chloroform-methanol (3/2), four times with 10 ml of chloroform-methanol-water (10/10/3), and four times with 10 ml of solvent E, which contains water-ethanol-diethylether-pyridine-NH₄OH (15/15/5/10/0.017) (29). The protein pellet was subjected to mild acid hydrolysis (40 mM trifluoroacetic acid treatment for 10 min). PNGase F treatment, overnight digestion with amylase from *Bacillus subtilis* (Fluka), and several washes with 50% methanol. Finally, O-glycans were released by alkali-induced β-elimination in the presence of sodium [1H]borotritide.

The labeled reduced O-glycans were separated by Bio-Gel P-2 chromatography (110- by 1-cm column) in 0.1 M acetic acid-1% butanol. Fractions of 400 μl were collected, and aliquots were taken for scintillation counting. Peak fractions were recovered and dried. To determine whether Dol-P-Glc is a donor for the reduced sugar in the O-glycan chain, samples were taken from each peak and hydrolyzed in 2 M HCl for 2 h at 90°C. After the samples were cooled on ice, acid was removed by evaporation. The radiolabeled sugars were chromatographed in a Dionex instrument, using a CarboPac MA1 column and hexose and alditol standards, as described previously (33).

**Nucleotide sequence accession numbers.** Nucleotide sequences for *TvDPM1* and derived amino acid sequences have been submitted to GenBank with accession number EU477838. All other *Trichomonas ALG5* genes have been previously annotated (see Table S1 in the supplemental material).

**RESULTS AND DISCUSSION**

*Trichomonas* membranes have ~300 times the Dol-P-Glc synthase activity of *Saccharomyces cerevisiae* membranes. *Trichomonas* membranes have ~300 times the Dol-P-Glc synthase activity of membranes prepared from *Saccharomyces* (Fig. 1). The Dol-P-Glc synthase activity of *Trichomonas* membranes is ~20 times the Dol-P-Man synthase activity of *Trichomonas* membranes, which was quite variable from experiment to experiment (Fig. 1). In contrast, the Dol-P-Man synthase activity of *Trichomonas* membranes is one-fifth that of *Saccharomyces* membranes, which in turn is ~75 times the Dol-P-Glc synthase activity of *Saccharomyces* membranes. In summary, *Trichomonas* membranes have a very high Dol-P-Glc synthase activity and a somewhat low Dol-P-Man synthase activity relative to *Saccharomyces* membranes.

Endogenous Dol-P-hexoses of *Trichomonas* contain 16 isoprenoid units. Dolichol chain length, which is determined by properties of the cis-prenyltransferase that catalyzes the formation of the dehydrodolichol pyrophosphate precursor, varies among the eukaryotes examined (16). Dolichols of mammals contain 15 to 23 isoprenoid units, those of *Saccharomyces cerevisiae* in logarithmic growth phase contain 14 to 18 isoprenoid units, and those of protists (*Plasmodium, Trypanosoma*, and *Leishmania* spp.) contain 11 or 12 isoprenoid units (2, 6, 19, 31).

The length of endogenous dolichol of *Trichomonas vaginalis* was estimated in three ways. First, Dol-P-Glc made from endogenous Dol-P of *Trichomonas* has the same mobility in TLC as *Saccharomyces* Dol-P-Glc, which is made from an endogenous Dol-P that predominantly contains 16 isoprenoid units (Fig. 2A). In contrast, the basis of *R. s.* Dol-P-Glc made from endogenous *Trichomonas* dolichol runs more slowly than that made with addition of exogenous Dol-P containing 19 isoprenoid units.

Second, dehydrodolichols produced in an in vitro assay of cis-prenyltransferase, in which *Trichomonas* membranes were incubated with radiolabeled isopentenyl pyrophosphate and exogenous farnesyl pyrophosphate, contain 15 or 16 isoprenoid units, like the predominant species of *Saccharomyces* (Fig. 2B).

Third, mass spectrometry showed that the predominant *Trichomonas* Dol-P-hexose, extracted from cultured parasites and fractionated on the acete form of a DEAE-cellulose column, contains 16 isoprenoid units. As shown in Fig. 2C, the observed [M-H]⁻ ion at m/z 1,350,043 (monoisotopic peak) is consistent with the expected value of 1,350,040 for the [M-H]⁻ ion of the Dol-P-hexose with 16 isoprenoid units.

**Five paralogous *Trichomonas* Alg5s have strong Dol-P-Glc synthase activities in transformed *Saccharomyces*.** Phylogenetic analyses of Alg5 and Dpm1 demonstrate three clades, which include Alg5s that have an N-terminal signal anchor, clade A Dpm1s that have a C-terminal transmembrane helix,
and clade B Dpm1s that lack a C-terminal transmembrane helix and are part of a multisubunit Dol-P-Man synthase complex (22). *Trichomonas* has five putative ALG5 genes (TvALG5A to TvALG5E), which encode proteins with N-terminal signal anchors that are part of the large Alg5 clade (Fig. 3; see Table S1 and Fig. S3 in the supplemental material). The five predicted *Trichomonas* Alg5 proteins are 325 to 337 amino acids long and show 52 to 80% identity to each other. Because these *Trichomonas* Alg5 proteins are much more similar to each other than to Alg5 proteins of other eukaryotes, they appear to be the product of numerous gene duplications after *Trichomonas* branched from the main eukaryotic tree. All five of these *Trichomonas* ALG5 genes complement a *Saccharomyces* alg5/H9004 mutant (Fig. 4A). In each case, the Dol-P-Glc synthase activity of the membranes from the *Saccharomyces* alg5/H9004 mutant transformed with TvALG5A to TvALG5E genes is greater than that of the wild-type *Saccharomyces* or that of the *alg5*Δ mutant expressing ScALG5 on the same promoter (Fig. 4A). The recombinant *Trichomonas* Dol-P-Glc synthases, which are encoded by TvALG5A to TvALG5E, also correct a carboxypeptidase Y N glycosylation defect in the *Saccharomyces* alg5Δ mutant in vivo (Fig. 4B).

While mRNAs for all five *Trichomonas* Dol-P-Glc synthase genes (TvALG5A to TvALG5E) were detected by reverse transcription-PCR with total RNA from *Trichomonas* trophozoites, those of TvALG5D and TvALG5E were by far the most abundant (Fig. 5). These results suggest that the abundant Dol-P-Glc synthase activity of *Trichomonas* membranes is caused by expression of multiple TvALG5A to TvALG5E gene products.

A deeply divergent *Trichomonas* DPM1 gene product has Dol-P-Man synthase activity in vitro. *Trichomonas* has two other genes (TvDPM1 and TvALG5G) that encode deeply divergent proteins, which are not members of the Alg5 clade or Dpm1 clades A or B (Fig. 3; see Fig. S3 in the supplemental material). TvALG5G encodes a 334-amino-acid protein that shows 19 to 24% identity to those encoded by TvALG5A to TvALG5E genes is greater than that of the wild-type *Saccharomyces* or that of the *alg5*Δ mutant expressing ScALG5 on the same promoter (Fig. 4A). The recombinant *Trichomonas* Dol-P-Glc synthases, which are encoded by TvALG5A to TvALG5E, also correct a carboxypeptidase Y N glycosylation defect in the *Saccharomyces* alg5Δ mutant in vivo (Fig. 4B).
weak identity with Tv\textit{ALG5G} (see Fig. S3 in the supplemental material). The Tv\textit{DPM1} gene, which was reconstructed from two overlapping protein predictions in the NCBI database, encodes a 345-amino-acid protein with a C-terminal transmembrane helix. With the exception of Tv\textit{Alg5G}, Tv\textit{DPM1} aligns poorly with all the other eukaryotic Dpm1s and Alg5s (see Fig. S3 in the supplemental material).

Tv\textit{Dpm1} shows Dol-P-Man synthase activity when expressed as a recombinant protein in \textit{E. coli} (Fig. 6). The Tv\textit{DPM1} gene product is slightly less active than \textit{Saccharomyces} Dol-P-Man synthase (Sc\textit{DPM1} gene product) expressed in \textit{E. coli}, and its activity is similar to the Dol-P-Glc synthase activity of the Tv\textit{ALG5E} gene product expressed in \textit{E. coli} (Fig. 6). The \textit{Trichomonas DPM1} gene product then is the most deeply divergent Dol-P-Man synthase identified to date. The Tv\textit{DPM1} mRNAs are much less abundant than those of the \textit{Trichomonas ALG5S} (Fig. 5), which is consistent with the variable Dol-P-Man synthase activity of \textit{Trichomonas} membranes in Fig. 1.

\textbf{Dol-P-Glc is incorporated into reducing and nonreducing sugars of \textit{Trichomonas} protein O-glycans.} Because \textit{Trichomonas} makes an N-glycan precursor that is not glucosylated (Man\textsubscript{n},GlcNAc\textsubscript{2},PP-dolichol) (10, 25), it is not clear what Dol-P-Glc is used for in \textit{Trichomonas} trophozoites. \textit{Trichomonas} membranes incubated with radiolabeled Dol-P-Glc incorporate radiolabeled Glc into glycolipids and glycoproteins. Control reactions, which demonstrate the dependence of this reaction on pH, on time, and on temperature, are shown in Fig. S2 in the supplemental material. Radiolabeled Glc, which is present in trace amounts in glycolipids extracted in chloroform-methanol-water (10/10/3), is also present in solution E extracts that contain \textit{Trichomonas} LPG (3, 8, 29). However, the counts were so small that we were unable to further characterize radiolabeled Glc from Dol-P-Glc in the \textit{Trichomonas} LPG fraction.

Radiolabeled Glc, which is associated with the glycoprotein pellet isolated from \textit{Trichomonas} membranes incubated with Dol-P-Glc, is not released by PNGase F or mild acid but is released by β-elimination. This result suggests that Dol-P-Glc is used for synthesis of glycoproteins with O-glycans but not those with N-glycans or O-phosphodiester-linked glycans. On a

\begin{figure}
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Phylogenetic reconstruction using the maximum likelihood method of representative Dol-P-Glc synthases (Alg5s) and Dol-P-Man synthases (Dpm1s). Branch lengths are proportionate to differences between sequences, and numbers at nodes indicate bootstrap values for 100 replicates. TvAlg5A to TvAlg5E, which result from gene duplications in \textit{Trichomonas}, belong to the Alg5 clade of other protists, fungi, and metazoa. TvAlg5G and TvDPM1 belong to no clade, while eukaryotic Dpm1s are split between two groups with (clade A) and without (clade B) a C-terminal transmembrane helix. In addition to \textit{Trichomonas vaginalis} (Tv), protists include \textit{Cryptosporidium parvum} (Cp), \textit{Plasmodium falciparum} (Pf), \textit{Toxoplasma gondii} (Tg), \textit{Leishmania major} (Lm), \textit{Trypanosoma cruzi} (Tc), \textit{Trypanosoma brucei} (Tb), \textit{Dictyostelium discoideum} (Dd), and \textit{Giardia lamblia} (Gl). Fungi include \textit{Saccharomyces cerevisiae} (Sc), \textit{Schizosaccharomyces pombe} (Sp), and \textit{Encephalitozoon cuniculi} (Ec), while metazoa include \textit{Homo sapiens} (Hs) and \textit{Drosophila melanogaster} (Dm). \textit{Arabidopsis thaliana} (At) is the single plant.}
\end{figure}
Bio-Gel P-2 sizing column, glycans containing radiolabeled Glc from the Dol-P-Glc incubation with *Trichomonas* membranes run in the void (large sugars) or near the total volume (single sugar) (Fig. 7A). Large sugars are predominantly composed of Glc, while the single sugar is glucitol (the reducing sugar) (Fig. 7B). As far as we know, this is the first time that Dol-P-Glc has been shown to be involved in the synthesis of O-glycans on glycoproteins.

**Major conclusions and unresolved questions.** Despite the fact that *Trichomonas* has no apparent need for either Dol-P-Glc or Dol-P-Man, *Trichomonas* membranes have synthase
activities for both activated sugars. *Trichomonas* has many *ALG5* genes, which are active in transformed *Saccharomyces* and likely account for the unusually large amount of Dol-P-Glc synthase activity of *Trichomonas* membranes. Conversely, *Trichomonas* has a deeply divergent *DPM1* gene, which encodes a Dol-P-Man synthase that likely is responsible for the Dol-P-Man synthase activity of *Trichomonas* membranes.

While we have not yet identified the uses for Dol-P-Man by *Trichomonas*, Dol-P-Glc may be used to make O-linked glycans in *Trichomonas* glycoproteins. This is similar to the use of Dol-P-Man by protein mannosyltransferases (PMTs) of yeast and protein O-mannosyltransferases (POMTs) of metazoa to make glycoproteins containing O-linked mannose (20, 35). Remarkably, *Trichomonas* has >30 genes which encode PMT- or POMT-like proteins that might use Dol-P-Glc or Dol-P-Man to make protein O-linked glycans (4).

While the use Dol-P-Glc to glucosylate N-glycan precursors in the lumen of the ER has been known for a long time (9–11), this is the first report of the use of Dol-P-Glc in the synthesis of O-linked glycans of glycoproteins. Dol-P-Glc may also be used to make ceramide (30). We speculate that *Trichomonas* will not be the only organism that uses Dol-P-Glc to make O-glycans on glycoproteins, and we do not rule out the possibility that *Trichomonas* uses Dol-P-Glc or Dol-P-Man to make glycolipids or LPG.

**ACKNOWLEDGMENTS**

This work was supported in part by NIH grants AI48082 (J.S.) and GM31318 (P.W.R.). Z.G. and the mass spectrometry facility in the Department of Biochemistry, Duke University Medical Center, were supported by a LIPID MAPS grant (GM-069338) from the National Institutes of Health. We are grateful to Ewa Szwieszewska (Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland) for providing dolichyl phosphate and Paula Magnelli (Boston University) for help with high-pH anion-exchange chromatography.

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