Cloning and functional expression of glycosyltransferases from parasitic protozoans by heterologous complementation in yeast: the dolichol phosphate mannose synthase from *Trypanosoma brucei brucei**

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The gene for the enzyme dolichol phosphate mannose (Dol-P-Man) synthase from the parasitic protozoan *Trypanosoma brucei brucei* (*T. brucei*) was cloned by screening a *T. brucei* cDNA library and then sequenced. The library was constructed in a yeast expression vector and the positive clone was identified by complementation of a temperature-sensitive defect in the yeast strain DPM 1-6 [Orlean, Albright and Robbins (1988) *J. Biol. Chem.* **263**, 17499–17507]. The insert of this clone displayed an open reading frame of 801 nucleotides coding for a putative protein of 267 amino acids. The deduced protein sequence showed an identity of 49% and a similarity of 69% with the published yeast sequence. Additional features of the *T. brucei* sequence are the presence of a putative signal sequence, a C-terminal transmembrane domain, a consensus sequence for phosphorylation by cAMP-dependent protein kinase and a

stretch of five nucleotides immediately upstream from the putative initiation codon that could function as a prokaryotic ribosome binding site. A consensus sequence for dolichol binding (FI/VXF/YXXIPXF/Y) found in the yeast protein could not be detected in the putative transmembrane domain of the *T. brucei* sequence. Biochemical characterization of the recombinant protein showed that it is functionally expressed in the yeast strain DPM 1-6 and *Escherichia coli*. In both constructs Dol-P-Man synthesis was shown in a cell-free system. Synthesis was stimulated by exogenous dolichol phosphate and inhibited by amphotericin. These results confirm that we have cloned the *T. brucei* Dol-P-Man synthase by heterologous complementation in yeast, an approach that might be applicable for other glycosyltransferases from various sources.

INTRODUCTION

Dolichol phosphate mannose (Dol-P-Man) is a central donor of activated mannoses for several glycosylation pathways. It participates in N-glycosylation of proteins, a pathway highly conserved among eukaryotes, by donating the last four mannosyl residues during the synthesis of the lipid-linked precursor oligosaccharide Dol-PP-GlcNAc₂Man₅Glc₃ [1]. N-glycosylation plays an important role in protein folding and quality control in the endoplasmic reticulum (ER) and is crucial for biosynthetic trafficking of proteins beyond the ER and throughout the secretory pathway [2]. In fungi such as yeast, Dol-P-Man supplies the first mannosyl residue that is O-glycosidically linked to protein [3,4]. It also is the mannose donor in the biosynthesis of glycosylphosphatidylinositol (GPI) membrane anchors [5]. This mechanism of anchoring protein in plasma membranes was first described in *T. brucei* [6,7] and, like N-glycosylation, has since been shown to be ubiquitous among eukaryotes. There is a growing body of evidence that these GPI anchors exhibit a variety of functions other than the mere anchoring of membrane proteins (reviewed in [8]) and in *Plasmodium falciparum*, the causative agent of malaria tropica, evidence has emerged that the

GPI moiety of merozoite surface proteins is a major fever toxin and might be responsible for exacerbating the effects of cerebral malaria [9,10].

Dol-P-Man synthase (EC 2.4.1.83), which synthesizes Dol-P-Man from GDP-Man and dolichol phosphate (Dol-P), plays a crucial role in these three pathways, which take place in the ER. The topology of Dol-P-Man biosynthesis and its further involvement in protein glycosylation is in itself an interesting problem because synthesis and utilization of this mannose donor apparently occur on opposite sides of the ER membrane [11,12]. The enzyme from *T. brucei* has been co-localized with ER markers [13], and the enzyme from yeast has been purified [14]. The yeast enzyme has been cloned and sequenced by Orlean et al. [15] from a temperature-sensitive (ts) yeast mutant, and it was shown that it is indeed an essential protein involved in N-, O-glycosylation and GPI anchor synthesis in yeast [4]. Furthermore they were able to complement glycosylation defects in mammalian cell lines with the cloned yeast gene [16,17].

Because glycosylation is a hallmark of surface proteins, these biosynthetic pathways play a particularly interesting role in the biology of parasites as surface antigens and their carbohydrate structures have to interact with the immune system of the host.

Abbreviations used: CEF, secondary chicken embryo fibroblasts; Dol-P, dolichol phosphate; Dol-P-Man, dolichol phosphate mannose; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; ts, temperature-sensitive.

* This work is dedicated to Dr. P. W. Robbins on the occasion of his 65th birthday, to whom R. T. Schwarz is particularly thankful for stimulating his interest in glycoconjugate biosynthesis.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Z54162.

In parasitic protozoans such as trypanosomes the anchoring of surface proteins through GPI anchors seems to be more widespread than in higher eukaryotes [18], thus making N-glycosylation and GPI anchor synthesis crucial for parasite survival. Taking into account the enormous medical and economical impact of protozoan parasites, there is a clear need to gain more knowledge about the enzymes involved in these pathways. Through recombinant DNA technology it should be possible to clone, express and subsequently characterize these enzymes, which are not readily accessible owing to the often limited availability of parasite material. It should be particularly interesting to elucidate key biochemical parameters as well as susceptibility towards inhibitors for these enzymes, especially in comparison with their mammalian counterparts, because further knowledge about these structures and their biosynthesis could have a profound impact on the development of vaccines and new antiparasitic drugs.

Because up-to-date sequence information on most of the enzymes involved in protein glycosylation is very limited it seems difficult to make assessments about conservation at the sequence level, although these proteins are conserved functionally across taxa.

We therefore decided to clone and characterize the Dol-P-Man synthase from *T. brucei*, an essential enzyme in protein glycosylation, by using the approach of heterologous complementation in yeast. Here we report the cloning and sequencing of the *T. brucei* Dol-P-Man synthase, the features of the deduced amino acid sequence and the result that the protozoan enzyme can be expressed in an active form by yeast and *Escherichia coli*.

MATERIALS AND METHODS

Chemicals

GDP-[³H]Man (20.5 Ci/mmol), [³⁵S]dATP (1000 Ci/mmol) and [³²P]dATP (800 Ci/mmol) were from NEN. Dolichol monophosphate (C80-105) was from Sigma, amphotycin was a gift from Troponwerke Colone, and Zymolyase 20000 was purchased from Seikagaku Corp., Tokyo, Japan. Restriction endonucleases and other DNA-modifying enzymes used in recombinant DNA experiments were from Boehringer-Mannheim, New England Biolabs or Stratagene and were used in accordance with the manufacturer's instructions.

Strains and media

The following *Saccharomyces cerevisiae* and *E. coli* strains were used in this work. The ts yeast strain dpm1-6 [Mat *a*; dpm1::Leu2; leu2-3,112; lys2-801; trp1-1; ura3-52;] and *E. coli* DH5 α harbouring the plasmid pDM6, which contains the structural gene (DPM1) for the yeast Dol-P-Man synthase, were a gift from Dr. P. Orlean [15]. YPH 499 [Mat *a*; ura3-52; lys2-801^{amber}; ade2-101^{ochre}; trp1- Δ 63; his3- Δ 200; leu2- Δ 1] (Stratagene) [19] was used as a 'wild-type control' in respect of Dol-P-Man synthase activity. *E. coli* strain XL1-blue (Stratagene) was used for subcloning and other standard recombinant DNA procedures. *S. cerevisiae* strains were grown in YPAD medium [1% (w/v) Bacto yeast extract, 2% (w/v) Bactopeptone, 2% (w/v) dextrose, 4 mg/l adenine] or SD medium (0.17% Bacto yeast nitrogen base and 2% dextrose containing the nutritional supplements necessary to complement strain auxotrophies or allow selection of transformants). *E. coli* strains were grown in Luria-Bertani medium supplemented with ampicillin (100 μ g/ml) when necessary.

Trypanosomes

Trypanosoma b. brucei of the Molteno Institute trypanozoon antigenic type 1.4 (variant clone 118) of *T. B.* strain 427 were purified from infected rat blood as described [20,21].

Recombinant DNA methods

All standard DNA techniques were performed as described by Sambrook et al. [22] unless otherwise stated. Plasmid isolation from *E. coli* was routinely performed with Quiaprep columns (Quiagen). Plasmid isolation from yeast followed the protocol of Hoffman and Winston [23]. Transformation of yeast was performed by the LiAc procedure [24]. Restriction fragments were separated by agarose gel electrophoresis in Tris/acetate buffer. Individual restriction fragments were isolated with the QIAquick Gel extraction Kit (Quiagen). Sequencing was performed by double-stranded dideoxy sequencing [25,26] with the Sequenase[®] sequencing Kit Version 2.0 from United States Biochemical. Computer analysis of sequence data was performed with the Heidelberg Unix Sequence Analysis Resources (HUSAR), release 3.0.

Construction of a cDNA library from *T. brucei*

Total RNA was prepared by the guanidinium thiocyanate/caesium chloride method [27]. Poly(A)⁺ RNA was isolated with the Poly(A)⁺ Quick[®] mRNA Purification Kit from Stratagene in accordance with the manufacturer's instructions. The cDNA synthesis was performed by using the Zap-cDNA synthesis kit from Stratagene for unidirectional cloning of cDNA inserts. The cDNA was ligated into the yeast expression vector pRS 416-Met [28], which had been digested with the restriction enzymes *Eco*RI and *Xho*I. This library was then transformed into *E. coli* DH5 α by electroporation (Electromax Gibco/BRL) and amplified.

Nucleic acid hybridization

Southern transfers were performed essentially as described [22], with the Boehringer Digoxigenin Labeling and Detection Kit for hybridization and detection. DNA fragments were separated on a 0.8% agarose gel in TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA], followed by alkaline denaturation. Nucleic acids were transferred to positively charged nylon membranes (Boehringer), which were processed in accordance with the manufacturer's instructions. Hybridization was performed at 60 °C overnight, with digoxigenin-labelled DNA fragments as probes, followed by two washes at room temperature in 2 $\times$ SSC/0.1% SDS and two additional washes at 68 °C in 0.1 $\times$ SSC/0.1% SDS for 15 min each (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate). Detection of hybridization signals was then performed in accordance with the manufacturer's instructions.

Preparation of lysates for assay of Dol-P-Man synthase

Dol-P-Man synthase activity was measured in crude lysates of yeast or *E. coli* cells. Exponential-phase yeast cultures (15 ml grown at either 26 or 37 °C to a D_{600} of 1, corresponding to 1.5 $\times 10^7$ cells/ml) were pelleted and washed in 1.0 ml of 1 M sorbitol/1 mM EDTA, pelleted and resuspended again in 1.0 ml of 1 M sorbitol/1 mM EDTA containing Zymolyase 20000 at a final concentration of 100 μ g/ml. These cell suspensions were incubated at 37 °C for 30 min, with light-microscopic control of cell wall degradation. Cells were then harvested by centrifugation

(1800 g, 10 min, 4 °C) and lysed by resuspension in 200 µl of TM buffer (50 mM Tris/HCl, pH 7.5, containing 5 mM MgCl₂ and 0.2 % 2-mercaptoethanol).

Lysates were assayed directly for Dol-P-Man synthase activity (30 µl for each assay, corresponding to 10⁸ cells). *E. coli* lysates were prepared as described in [15]. *T. brucei* parasites, purified from infected rat blood, were adjusted to the same cell density as the yeast cells and lysed in TM buffer.

Assay of Dol-P-Man synthase

Enzyme assays were performed as described [3]. Briefly, incubation mixtures contained 5 µl of GDP-[³H]Man (0.1 mCi/ml; 0.5 µCi), 8 µl of Dol-P (2 mg/ml dispersed in 0.5 % Triton X-100 by sonication), amphomycin (final concentration 1 mg/ml in some samples) and water to give a final volume of 70 µl. Reactions were started by the addition of 30 µl of cell lysates and incubated at 37 °C (the non-permissive temperature) for 10 min. Incubations were terminated after 10 min by the addition of 2 ml of ice-cold chloroform/methanol (2:1, v/v) and centrifuged (1500 g, 5 min, 4 °C). The pellet was extracted again with 500 µl of chloroform/methanol. Both chloroform/methanol phases were combined and subjected to repeated Folch washing [3]. An aliquot of the Folch-washed material was taken for determination of the radioactivity in the organic phase by scintillation counting. Samples of the radiolabelled, chloroform/methanol-extractable reaction products were analysed by TLC on Silica 60 plates (Merck) with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as solvent, with Dol-P-Man prepared from chicken embryo fibroblasts as a reference [29]. Plates were screened for radioactivity with a Berthold LB 2842 Automatic TLC-Linear Analyzer.

To demonstrate the nature of the lipid-linked radioactivity, the radiolabelled material was purified by DEAE-cellulose chromatography and subjected to mild acid hydrolysis (1 M HCl, 15 min, 50 °C) as described by McDowell and Schwarz [30]. The reaction products were analysed by HPLC chromatography (Dionex) on a CarboPack PA1 column by isocratic elution with 15 mM NaOH.

RESULTS

Isolation of a *T. brucei* cDNA fragment complementing the ts defect of the yeast strain DPM1-6

Our strategy to clone the Dol-P-Man synthase gene from *T. brucei* was the use of heterologous complementation in yeast because N-glycosylation and GPI anchor synthesis are evolutionally conserved pathways and thus the corresponding enzyme activities should be present in both organisms. The yeast strain DPM 1-6 was transformed with the *T. brucei* cDNA library as described in the Materials and methods section, after which 125000 recombinant yeast clones were plated and grown at the permissive temperature of 27 °C, replica plated and incubated at the non-permissive temperature of 37 °C. Initially, 98 clones were obtained growing at 37 °C. To eliminate false positives, mainly revertants of the ts phenotype, these clones were plated on medium containing 5-fluoro-orotic acid at 27 °C to screen for loss of plasmid [31]. Colonies were then streaked out again and incubated at 37 °C. The 12 clones that lost their ability to grow at the elevated temperature were analysed further.

Recombinant plasmids were isolated, amplified in *E. coli* and used to retransform the ts strain DPM 1-6. One of these clones (clone pTB 28) was repeatedly able to rescue the ts phenotype of DPM 1-6 and was thus chosen for sequencing and biochemical analysis.

Figure 1 Physical map of the cDNA clone pTB 28

The restriction sites that were used for subcloning are indicated; the abbreviations are: A, *AccI*; Bc, *BclI*; Bs, *BsaI*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; X, *XhoI*. The position of the open reading frame (ORF) is represented by the hatched box, with the direction of translation indicated by an arrow.

1	gaattcggcagcaggggaacagggcggtgtgctagcagatgtaaaagataattgtacta	60
61	caccagtttattccacgtattttttaccttctctgtagttccatcggggtttcttctctc	120
121	agaagtgcacaaagagaaggagaatgacaaacggaagagcggaagaaattttgttta	180
181	ggtaaaacagctacaatacaaggaagtagaacaatggcggtgaagtattccatcattgtt	240
	*****MetAlaValLysTyrSerIleIleVal	
241	ccagcctacaaggatgtgggaacttgagccactacaagcagggttgatcgctcggt	300
	ProAlaTyrLysGluCysGlyAsnLeuSerHisLeuGlnAlaGlyValAspArgLeuAla	
301	gacgatgtgtttttccaaatgaagtggaaatggaatgttagacgataactccagagac	360
	AspAspGlyPheSerLysAsnGluValGluMetValIleValAspAsnSerArgAsp	
361	ggttcgttgtaagtgttgaggaaatccgaatgaaggctacgggtgctgacgaatg	420
	GlySerValGluValValGluLysValArgAsnGluGlyTyrGlyValArgIleGluVal	
421	aggacgaacatcggtgtgagcagtcagtaattcatggcatttccgtatccaagga	480
	ArgThrAsnAspArgGlyLeuSerSerAlaValIleHisGlyIleSerValSerLysGly	
481	agcttcattctcgttaattgagtcgggaccttcagcaccacacaaacagtagctgtt	540
	SerPheIleLeuValMetAspAlaAspLeuGlnHisProProLysThrValProCysLeu	
541	ctgagggcacttgaacaaactggtgttgagttcgtttgtggaactcgttacggagctgt	600
	LeuArgAlaLeuGluLysProGlyValGluPheValCysGlyThrArgTyrGlyAlaGly	
601	gttgaattgacaggaactggcggttgccacctgcttcattatcggtgggtgcgcgaac	660
	ValGluIleAspLysAspTrpProLeuHisArgArgPheIleSerTrpGlyAlaArgLeu	
661	ctggcgagggcacttacaccactgagtgatcccatcggtgtgtttttgtgctctcggt	720
	LeuAlaArgProLeuThrProLeuSerAspProMetSerGlyPhePheGlyProSerArg	
721	tcgattgttttcaacggcggtcgagaagttgtgaacctattgggtacaaaatcgacta	780
	SerMetPhePheAsnGlyGlyArgGluValValAsnProIleGlyTyrLysIleAlaLeu	
781	gagctatttggtaagtgtgctgttcgtaaatatgaggaaagttggaatttaactttgctcg	840
	GluLeuPheValLysCysAlaValArgLysTyrGluGluValGlyPheAsnPheAlaAla	
841	cgcactgtagcgagagtaagttaacaggttaagtgatcatcaattacttagagcacctg	900
	ArgThrValGlyGluSerLysLeuThrGlyLysValIleIleAsnTyrLeuGluHisLeu	
901	aagctcctctacttttactgtgtatggcaccgcgctcacgggttctactcgtgctgtgcc	960
	LysLeuLeuTyrPheTyrValTyrGlyThrAlaLeuThrGlySerThrArgAlaValAla	
961	ggcttatttttactgtttttacattctctttagcacacacctttcccttctgttagttt	1020
	GlyLeuPhePheThrValPheThrPhePheSerThrHisLeuSerLeuAla	
1021	gttgtaaaagagattgttactctgtggtaccagctggggagaaaagagaacccctggggcg	1080
1081	tactcctaccacatctcctaataaacctttctgttgcctctaacgcactccactgggata	1140
1141	tatttgtgtgcgaaaacagtcgctgtttttccgtgctatttttttaaaaaaaaaaaaaa	1200
1201	aaa	1260
1261	aaaaaaaaaaaaaaaaaactgcacctcgag	1288

Figure 2 DNA and derived amino acid sequence of the *T. brucei* Dol-P-Man synthase gene

The predicted N-terminal cleavage point for the signal peptidase is indicated by a triangle; the potential C-terminal transmembrane domain is underlined. The potential phosphorylation site (Arg-Arg-Phe-Ile-Ser) is underlined twice and the DNA sequence AAGGA, which could serve as a prokaryotic ribosome binding site, is marked with asterisks.

Sequence analysis of the *T. brucei* Dol-P-Man synthase gene

Restriction analysis of clone pTB 28 revealed a cDNA insert of 1.3 kb. Figure 1 shows the physical map of the insert with the restriction sites used for subcloning. The DNA sequences of both strands were determined by double-strand sequencing after generation of subclones in SK vectors. For regions that were not readily accessible by standard subcloning, specific DNA primers were synthesized to span those gaps.

Figure 3 Hydrophobicity plot of the deduced protein sequence of clone pTB 28

In this graphic presentation, a hydrophobicity index above 1.6 defines potential transmembrane domains. For this analysis a window of 9 was used.

An open reading frame of 801 base pairs was identified revealing a protein sequence of 267 amino acids with a calculated molecular mass of 29 559 kDa and an isoelectric point of 8.51. Figure 2 shows the cDNA and derived amino acid sequence of the putative *T. brucei* Dol-P-Man synthase. Computer-guided sequence analysis of the predicted protein sequence revealed an N-terminal stretch of amino acids that could serve as a signal sequence with a predicted cleavage site after amino acid residue 30 as described by Von Heijne [32]. The hydrophobic sequence at the C-terminus shows the characteristics of a transmembrane domain described by Kyte and Doolittle [33]. No consensus sequence (Asn-Xaa-Ser/Thr) for N-glycosylation was detected.

The sequence also contains a putative phosphorylation sequence for cAMP-dependent protein kinase around Ser-145. The amino acid sequence surrounding this serine fulfils the requirements for such a consensus sequence [34].

The *T. brucei* cDNA clone does not contain the postulated 'dolichol recognition sequence' FI/VXF/YXXIPFXF/Y [35,36] which, in the yeast sequence, is located in the C-terminal transmembrane domain. The putative membrane-spanning region of the *T. brucei* C-terminus does not contain a stretch of 11 amino acids resembling this consensus. An isoleucine that has been found at position 7 in all consensus sequences so far, and has thus been postulated to be invariant, is not present in the putative transmembrane domain of the *T. brucei* Dol-P-Man synthase. Direct sequence comparison between the yeast and the *T. brucei* protein sequence shows an identity of 49% and a similarity of 69%, placing the putative phosphorylation site at an identical position. The hydrophobicity plot of pTB 28 (Figure 3) shows an identical distribution of hydrophobic and hydrophilic regions for the yeast and the *T. brucei* protein, which becomes strikingly apparent when both plots are superimposed.

Southern blot analysis of clone pTB 28

To confirm that the cDNA insert of clone pTB 28 is of *T. brucei* origin, and to obtain a preliminary assessment of the copy number, Southern blots were performed under stringent conditions as described in the Materials and methods section. When *Bam*HI digests of genomic DNA from the protozoan parasites *T. brucei*, *P. falciparum* and *Toxoplasma gondii* as well as DNA from a mammalian cell line (VERO) were probed with the digoxigenin-labelled cDNA insert, a band in the upper 20 kb

Figure 4 Southern blot analysis of genomic digests probed with the digoxigenin-labelled insert of clone pTB 28

(A) *Bam*HI-digested DNA of *T. gondii* (lane 1), *P. falciparum* (lane 2), *T. brucei* (lane 3) or VERO-DNA (lane 4), with clone pTB 28 (undigested) as positive control (lane 5). (B) *T. brucei* DNA digested with *Bam*HI (lane 1) or *Hind*III (lane 2), plus undigested pTB 28 (lane 3). M, size markers; the sizes of the DNA marker fragments are indicated in kb at the left of each blot.

range could be detected with the *T. brucei* DNA only (Figure 4A). When *Bam*HI and *Hind*III digests of *T. brucei* DNA were hybridized under the same conditions (Figure 4B), two bands could be detected in the *Hind*III digest, confirming the physical map and suggesting that the Dol-P-Man synthase is a single-copy gene in *T. brucei*.

The cloned *T. brucei* Dol-P-Man synthase is active in yeast and *E. coli* lysates

The capability of the cDNA cloned from *T. brucei* to complement repeatedly the ts phenotype of the yeast strain DPM 1-6 strongly suggests in itself that the coding sequence does indeed represent the enzyme Dol-P-Man synthase. To confirm this hypothesis we performed enzyme assays as described in the Materials and methods section. Yeast cells were grown at the permissive temperature and lysates were assayed for Dol-P-Man synthase activity at 37 °C.

When the ts strain DPM 1-6 was assayed, only minute amounts of radioactivity could be detected in the chloroform/methanol phase. The same result was obtained with DPM 1-6 harbouring the plasmid pRS 416 Met only. The yeast strain YPH 499 and secondary chicken embryo fibroblasts (CEF) used as reference produced significant amounts of radioactivity when supplemented with external Dol-P. The ts yeast strain DPM 1-6 transformed with either pDM 6, the yeast gene, or pTB 28 produced significant amounts of chloroform/methanol-extractable radiolabelled material. The reaction could be stimulated by the addition of external Dol-P and inhibited by the addition of amphotycin, a specific inhibitor of Dol-P-Man synthase [5,37]. A similar picture emerged when *E. coli* harbouring pRS 426 Met, pDM6 or pTB28 were assayed. When *T. brucei* lysates were assayed in the same manner, incorporation of radioactivity was obtained. The results of these assays are summarized in Table 1.

To confirm the identity of the radiolabelled reaction products, chloroform/methanol-extractable glycolipids were analysed by TLC. Dol-P-Man from CEF was included as an internal standard. Figure 5 shows the results of these analyses. In all positive

Table 1 Summary of Dol-P-Man synthase assays performed with various constructs

Assays were performed as described in the Materials and methods section. The relative Dol-P-Man synthase activity is expressed as c.p.m. monitored in the organic phase after chloroform/methanol extraction.

Source	Dolichol-P	Amphotycin	Dol-P-Man synthase activity (c.p.m.)
<i>T. brucei</i>	+	—	34 000
CEF	+	—	5700
CEF	+	+	125
Yeast:			
YPH499	+	—	1587
DPM 1-6	+	—	99
DPM 1-6 [pDM6]	+	—	27 000
DPM 1-6 [pDM6]	—	—	980
DPM 1-6 [pTb28]	+	—	11 000
DPM 1-6 [pTb28]	—	—	800
DPM 1-6 [pTb28]	—	+	60
DPM 1-6 [pTb28]	+	+	680
<i>E. coli</i> :			
[pRS416MET]	+	—	99
[pDM6]	+	—	3050
[pTb28]	+	—	11 406

Figure 5 TLC analysis of Dol-P-Man synthase assays

Assays and TLC analysis were performed as described in the Materials and methods section. The panels represent assays as follows: (A) DPM 1-6 harbouring the plasmid pRS 416MET; (B) DPM 1-6 harbouring the cDNA clone pTb 28; (C) *E. coli* harbouring pRS 416 MET; (D) *E. coli* harbouring clone pTb 28. Exogenous Dol-P was added in these experiments. The position of Dol-P-Man from CEF is indicated by an arrow in (B).

reactions a peak of radiolabelled material was detected, migrating with the CEF standard.

To confirm that the radioactivity in the chloroform/methanol extractable compound was mannose and thus verify the identity of the radiolabelled peak as Dol-P-Man, the reaction products were purified by DEAE-cellulose chromatography followed by mild acid hydrolysis. This treatment liberates the carbohydrate moiety from the Dol-P, which then can be analysed by HPAEC Chromatography (Dionex). Figure 6 shows the result of such an analysis for the reaction product produced by the *T. brucei* cDNA fragment in DPM 1-6 at 37 °C. After hydrolysis the

Figure 6 Dionex HPAEC analysis of radiolabelled carbohydrates from the Dol-P-Man synthase assay of clone pTb 28 after mild acid hydrolysis

The abbreviation used is: inj, injection peak.

radioactivity co-elutes on HPAEC chromatography with an internal mannose standard, clearly demonstrating that the product of these enzyme assays is Dol-P-Man and thus the open reading frame of our *T. brucei* cDNA clone codes for a functional Dol-P-Man synthase.

DISCUSSION

We have cloned the gene for the *Trypanosoma b. brucei* Dol-P-Man synthase from an expression cDNA library by heterologous complementation of a ts defect in the yeast strain DPM1-6. The cDNA clone pTb 28 harbours the coding sequence for this enzyme because not only was it able to complement this defect, it was also shown to exhibit Dol-P-Man synthase activity *in vitro*. Furthermore a direct sequence comparison with the cloned yeast gene showed a coding sequence of identical length (801 nucleotides, 267 amino acid residues) with a predicted signal sequence and a C-terminal stretch of hydrophobic amino acids that could function as a transmembrane domain. Another striking similarity is the fact that both recombinant proteins are functional in *E. coli*. Because *E. coli* is able to utilize some yeast promoters [28], the insert might be transcribed from the MET 25 promoter of the cloning vector used. The construction of this vector also requires translation from endogenous ATG codons of cloned cDNA inserts because the β Gal fusion peptide of the original pRS vector has been deleted [28]. We therefore examined the regions immediately upstream of the predicted initiation codon of the *T. brucei* cDNA insert and found a putative ribosome binding site (Shine–Dalgarno Sequence) [38] of the sequence AAGGA with a spacing of nine nucleotides between this sequence and the ATG codon, thus fulfilling the criteria for binding of *E. coli* 30 S ribosomal subunits [39,40].

Another issue that has to be addressed in the context of significant enzyme activity in *E. coli* is the presence of a putative phosphorylation site in both the yeast and the *T. brucei* proteins (*T. brucei*, residues 141–146; yeast, residues 136–141). Because both enzymes are active in *E. coli*, phosphorylation is clearly not needed for enzyme function in itself. It thus can only be speculated that this site might be used for modulation of the enzyme activity that has been discussed for the mammalian enzyme [41]. Site-directed mutagenesis and phosphorylation *in vitro* might therefore give more insight into the validity and relevance of this phosphorylation site.

Dol-P-Man synthase belongs to a class of enzymes that have dolichol derivatives as substrates. From sequence analyses of

several such enzymes a so-called dolichol recognition sequence was identified and a consensus sequence for dolichol binding (FI/VXF/YXXIPXF/Y) was postulated [35,36]. This hydrophobic sequence, which is positioned within the C-terminal transmembrane domain of the yeast protein, could not be detected in the *T. brucei* sequence. This striking finding could be explicable in light of the finding of two groups that in the yeast Dol-P-Man synthase this sequence is not essential for enzyme activity *in vivo* and growth. Deletion of this region decreased the enzyme activity *in vitro* but a significant residual activity still remained that was able to allow growth of recombinant yeast clones as well as protein glycosylation in *S. cerevisiae* [42,43]. In contrast with these findings it was shown by Datta and Lehrman [44] that this region is essential for activity of a mammalian glycosyltransferase, the hamster GlcNAc-1-phosphate transferase, which, in contrast with the corresponding yeast enzyme, possesses two putative dolichol recognition sequences, both essential for enzyme function.

The localization or distribution of Dol-P-Man synthase in the secretory pathway is not fully understood. If the assumed luminal orientation of the enzyme is correct, both proteins lack a significant cytoplasmic tail and thus a retention signal of the KDEL or KKXX type [45,46]. Although localization of the *T. brucei* enzyme in the ER has been postulated [13], the overall distribution throughout the secretory pathway still remains unclear because proteins lacking such retention signals are believed to be transported through the secretory pathway with the bulk flow of materials. In addition, details of the topology of glycosyltransferases involved in dolichol-mediated oligosaccharide biosynthesis and subsequent protein glycosylation, including the molecular mechanisms for the translocation of lipid-linked sugars across the ER membrane, still remain speculative. If the current models for dolichol-mediated glycosylation are correct, the newly synthesized Dol-P-Man must cross the ER membrane to function as a mannose donor during N-glycosylation or GPI anchor biosynthesis. Rush and Waechter [47] showed recently that in sealed microsomal vesicles a water-soluble Dol-P-Man analogue was imported into these vesicles and that this uptake exhibited the characteristics of a protein-mediated transport, thus indicating that specific proteins might indeed be involved in the translocation of Dol-P-Man across the ER membrane. Highly purified recombinant enzymes should provide the tools for addressing these questions, for example by raising specific antibodies for high-resolution immuno-gold electronmicroscopy.

In addition to these purely biochemical approaches to enzyme function, purified enzyme preparations and assays *in vivo* by growth inhibition could also provide valuable tools for designing new antiparasitic drugs by exploiting differences between parasite enzymes and their mammalian (human) counterparts, some of which have already been discussed for the yeast and human Dol-P-Man synthase [43].

The results presented here demonstrate that the strategy of heterologous complementation in yeast proved to be a straightforward approach that could be exploited for other enzymes involved in protein glycosylation from various sources such as parasites, to obtain these proteins in sufficient quantities to address the questions discussed above.

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