Biosynthesis of glycosylphosphatidylinositols of *Plasmodium falciparum* in a cell-free incubation system: inositol acylation is needed for mannosylation of glycosylphosphatidylinositols

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The structures of glycosylphosphatidylinositols (GPIs) in *Plasmodium* have been described [Gerold, Schuppert and Schwarz (1994) J. Biol. Chem. **269**, 2597–2606]. A detailed understanding of GPI synthesis in *Plasmodium* is a prerequisite for identifying differences present in biosynthetic pathways of parasites and host cells. A comparison of the biosynthetic pathway of GPIs has revealed differences between mammalian cells and parasitic protozoans. A cell-free incubation system prepared from asexual erythrocytic stages of *Plasmodium falciparum*, the causative agent of malaria in humans, is capable of synthesizing the same spectrum of GPIs as that found in metabolically labelled parasites. The formation of mannosylated GPIs in the cell-free system is shown to be inhibited by GTP and, unexpectedly, micromolar concentrations of GDP-Man. Lower concentrations of GDP-Man affect the spectrum of GPIs synthesized. The inositol ring of GPIs of *P. falciparum* is modified by an acyl group. The preferred donor of this fatty acid at the inositol ring is myristoyl-CoA. Inositol acylation has to precede the mannosylation of GPIs because, in the absence of acyl-CoA or CoA, mannosylated GPIs were not detected. Inositol myristoylation is a unique feature of plasmodial GPIs and thus might provide a potential target for drug therapy.

Key words: malarial toxin, nucleotide sugar, parasitic protozoa.

INTRODUCTION

Plasmodium falciparum malaria is still the most important disease caused by a parasitic protozoan. It infects approx. 250 million people worldwide and is responsible for approx. 2 million deaths each year. Glycosylphosphatidylinositols (GPIs) of the parasite have been identified as toxins affecting host cell immune responses [1].

The biosynthesis of GPIs is a complex process involving inositol acylation, the successive transfer of sugars from activated sugar donors and various numbers of ethanolamine phosphates to the glycan core of these glycolipids. Most reactions involved in the assembly of GPIs are similar for all eukaryotic cells (reviewed in [2–8]). However, some aspects of GPI biosynthesis vary significantly between different species. The first steps in the biosynthesis of GPIs lead to the formation of a glucosaminyl-inositol-phosphate–lipid intermediate by the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to an inositol phosphate lipid and the immediate de-N-acetylation of the GlcNAc. By using different chemically synthesized GlcNAc-PtdIns analogues in a de-N-acetylase assay, differences in substrate specificities have been established between the trypanosomal enzyme and a mammalian enzyme [9]. In mammalian cells and yeast, intermediates of GPI biosynthesis receive a palmitoyl residue on the inositol ring before the attachment of the first mannose residue of the evolutionarily conserved trimannosyl core glycan, whereas in trypanosomes the first mannosylation step must precede palmitoylation of the inositol ring [10,11]. The donor for the acyl chain on the inositol ring is palmitoyl-CoA in yeast [12], whereas trypanosomes seem to use another palmitoyl source [13,14]. Controversial evidence about the donor of the palmitoyl chain in different mammalian cell lines has been described: palmitoyl-CoA [15] and a phospholipid [16] have been discussed as potential donors.

The mannosylation of early intermediates of GPI biosynthesis leading to the evolutionarily conserved trimannosyl core glycan involves the transfer of mannosyl residues from dolichol-phosphate-mannose as the donor in all systems investigated so far, including *P. falciparum* [17] [2–8]. Attaching ethanolamine phosphate from phosphatidylethanolamine as donor to the distal end of the trimannosyl core glycan of GPI-anchor biosynthesis intermediates is the final prerequisite for the transfer of GPIs to protein. Mammalian and yeast GPIs receive an additional ethanolamine phosphate attached to the innermost mannose residue [18–20], whereas the core glycan of GPI anchors in parasitic protozoans is not substituted with additional ethanolamine phosphate moieties (reviewed in [3]). This finding might explain the differences in susceptibility between mammalian cells and yeast on the one hand and protozoans on the other towards a novel, very potent inhibitor of GPI biosynthesis, YW 3548 [21]. Differences in the susceptibility to other inhibitors of GPI biosynthesis have been described for protozoans, yeast and mammalian cells [22–24]. This points to the possibility of developing parasite-specific inhibitors of GPI biosynthesis with therapeutic potential.

Many insights into GPI biosynthesis come from studies *in vitro* making use of cell-free incubation systems. These systems synthesize and accumulate biosynthetic intermediates, which are not found in significant amounts by using metabolic labelling techniques [25–27]. In addition, cell-free systems are an important tool for testing potential inhibitors affecting GPI biosynthesis (reviewed in [3]).

We have shown previously that lysates prepared from trophozoites of the human malaria parasite *P. falciparum* were able to

Abbreviation used: GPI, glycosylphosphatidylinositol.

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synthesize a spectrum of GPIs [17] matching those described for metabolically labelled intact parasites [28]. This spectrum varies during the intraerythrocytic cycle of the parasite [29]. On the basis of structural data, we proposed a pathway for GPI biosynthesis in *P. falciparum* that resembled the sequence of GPI synthesis described for mammalian cells [28]. GPIs are the major type of glycosylation found in *P. falciparum* [30]. To investigate parasite-specific inhibitors able to discriminate between parasite and mammalian GPI biosynthesis, it is of importance to understand this essential pathway in malaria parasites in more detail. Here we report on the use of cell-free incubation studies to investigate the biosynthesis of malarial GPIs. We show that GPI synthesis is independent of ATP, that inositol acylation has to precede the first mannosylation step in GPI biosynthesis and that the concentration of GDP-Man added to the system influences the spectrum of GPIs synthesized, especially the addition of the fourth mannose to the plasmodial GPI core glycan.

MATERIALS AND METHODS

Materials

D-[6-^{3}H]Glucosamine hydrochloride (26.0 Ci/mmol), D-[2-^{3}H]mannose (18.0 Ci/mmol), UDP-[6-^{3}H]GlcNAc (18.9 Ci/mmol) and GDP-[2-^{3}H]mannose (15.1 Ci/mmol) were purchased from Amersham (Braunschweig, Germany). Acyl-CoAs of different chain lengths were from Sigma (Deisenhofen, Germany). PtdIns-specific phospholipase C (*Bacillus cereus*) was from Boehringer Mannheim (Mannheim, Germany). Ion-exchange resins were of analytical grade and were purchased from Bio-Rad (München, Germany). All sugar and lipid standards were obtained from Sigma. All solvents used were of analytical or HPLC grade.

Parasites

P. falciparum strain FCBR was obtained from Dr. B. Enders (Behring Co., Marburg, Germany). It was maintained as described previously [29]. The development and multiplication of plasmodial cultures were followed by microscopic evaluation of Giemsa-stained smears. Parasite cultures were routinely checked for contamination with *Mycoplasma*.

Preparation of cell-free incubation systems from *P. falciparum* trophozoites

Washed parasites (30–40 h after invasion) were harvested by lysis with saponin [28]. Parasite lysates were prepared essentially as described previously [25]. In brief, approx. 5×10^9 parasites were lysed hypotonically and homogenized with 20 strokes of a Dounce homogenizer. An equal volume of double-isotonic-strength buffer was added. This preparation was designated parasite lysate. All experiments involving parasite lysates were performed with freshly prepared lysates. Parasite membranes were prepared by centrifuging parasite lysates at 15000 ***g*** for 10 min at 4 °C. Subsequently the membranes were washed in Hepes-buffered saline [100 mM Na/Hepes (pH 7.4)/50 mM KCl/10 mM $MgCl_2$/100 μM tosyl-lysylchloromethane ('TLCK')/1 μg/ml leupeptin] and stored at 5×10^9 parasite equivalents/ml for less than 8 weeks at −80 °C in the same buffer containing 20 % (v/v) glycerol. Before use, these membranes were washed twice with Hepes-buffered saline and resuspended in the same buffer containing 5 mM $MnCl_2$.

Labelling of parasite glycolipids *in vivo* and *in vitro*

Metabolic labelling of parasite cultures (*in vivo*) with tritiated glucosamine was performed as described [28]. For cell-free labelling, approx. 5×10^8 parasite equivalents, processed as parasite lysates or membrane preparations, were supplemented with 1 mM CoA (CoA), 1 mM ATP, 1 mM UDP-GlcNAc and 2 μCi of GDP-[2-^{3}H]Man. Incubations were performed for 45–90 min at 37 °C. Labelling with 3 μCi of UDP-[6-^{3}H]GlcNAc was performed after the addition of 1 mM CoA, 1 mM ATP and 1 mM GDP-Man to parasite lysates or membranes for 45–90 min at 37 °C.

For some experiments, parasite membranes were labelled in the presence of 0.1 mM acyl-CoA of different acyl chain lengths without additional supplements. For other experiments, parasite membranes were supplemented or not with 10 μM GDP-Man, 1 mM CoA or GDP-Man plus CoA. The effect of different concentrations of CoA was tested by using membrane preparations labelled with UDP-[6-^{3}H]GlcNAc without additional supplements.

For investigating the synthesis of non-mannosylated GPIs, 5 mM EDTA, 1 mM CoA and 1 mM ATP were added to parasite lysates in the absence of GDP-Man. EDTA blocks the formation of dolichol-phosphate-mannose [26]. Different concentrations of GDP-Man were tested for their effect on GPI biosynthesis in the presence of 1 mM ATP, 1 mM CoA and 3 μCi of UDP-[6-^{3}H]GlcNAc.

Extraction and purification of lipids

Glycolipids were extracted with chloroform/methanol/water (10:10:3, by vol.), as described [29]. The extracted glycolipids were dried in a Speedvac concentrator (Savant), subjected to repeated 'Folch' partitions, and finally partitioned between water and water-saturated butan-1-ol. Washed glycolipid extracts were analysed on silica-gel 60 TLC plates (Merck) with chloroform/methanol/water (4:4:1, by vol.) as the solvent system. After chromatography the plates were dried and scanned for radioactivity with a Berthold LB 2842 automatic TLC scanner or analysed by a BAS-1000 Bio-Imaging Analyser (Fuji Film). The areas of the TLC plates corresponding to the peaks of radioactivity were scraped off and glycolipids were eluted from the silica with chloroform/methanol/water (10:10:3, by vol.) [28].

Characterization of glycolipids

TLC-purified glycolipids were identified as GPIs by treatments including PtdIns-specific phospholipase C, GPI-specific phospholipase D and deamination with nitrous acid as described previously [28].

Analysis of the neutral core glycans

Neutral core glycans were prepared from TLC-purified glycolipids by dephosphorylation, deamination and reduction as described previously [28]. The desalted core glycans were analysed by high-pH anion-exchange chromatography (Dionex Corp., Idstein, Germany) [28].

RESULTS

Plasmodial cell-free systems synthesize the GPIs found in metabolically labelled parasites

Parasites were released from host cells by lysis with saponin. Parasite lysates and membranes were prepared from 5×10^8

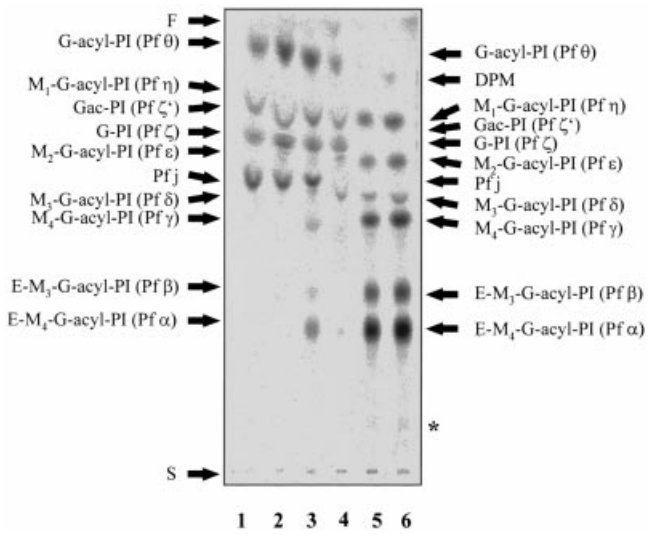

Figure 1 Glycolipids labelled in lysates or membranes prepared from asexual stages of *P. falciparum*

Parasite lysates (lanes 1, 3 and 5) or membrane preparations (lanes 2, 4 and 6), supplemented with CoA and ATP, were labelled with UDP-*N*-acetyl-[^{3}H]GlcN in the absence of GDP-Man (lanes 1 and 2) or in the presence of GDP-Man (lanes 3 and 4), or they were labelled with GDP-[^{3}H]Man in the presence of UDP-GlcNAc (lanes 5 and 6). Glycolipids were extracted by chloroform/methanol/water (10:10:3, by vol.) and analysed by TLC. Glycolipid extracts containing *P. falciparum* GPIs metabolically labelled with [^{3}H]GlcN or [^{3}H]Man were analysed in parallel. Radioactivity was detected with a BAS-1000 Bio-Imaging Analyser (Fuji Film). Eight glucosamine-labelled GPIs were identified in parasite lysates and membrane preparations. They were designated as follows: Pf α, Pf β, Pf γ, Pf δ, Pf ϵ, Pf ζ, Pf η and Pf θ. Pf ζ' was characterized as GlcNAc-PtdIns. Abbreviations: DPM, dolichol-phosphate-mannose; PI, PtdIns; G, GlcN; Gac, GlcNAc; M, mannose; E, ethanolamine phosphate; *, uncharacterized glycolipid; S, migration start; F, solvent front. Pf j was identified as a non-GPI glycolipid.

Table 1 Identification of GlcNAc-PtdIns synthesized by parasite lysates

Parasite lysates were labelled with UDP-[6-^{3}H]GlcNac in the presence of EDTA and the absence of exogenous GDP-Man. Glycolipids were extracted, divided into five aliquots and treated with PtdIns-specific phospholipase C (PI-PLC), GPI-specific phospholipase D (GPI-PLD) and nitrous acid. (NaCl was the negative control for treatment with nitrous acid.) After phase partitioning with butanol/water, the organic phases were analysed by TLC. Radioactivity was analysed by a Berthold TLC scanner (LB 1842) and the cleavage efficiency of each single peak was assessed with Chroma-peak integration software (Berthold).

		(Radioactive counts in the organic phase)				
Molecule	R_F	Control	GPI-PLD	PI-PLC	NaCl	Nitrous acid
Pf j	0.6	3143	2727	4312	2898	3543
GlcN-PI	0.7	8505	286	897	7315	3522
GlcNAc-PI	0.77	4834	5395	1966	3314	4208
GlcN-acyl-PI	0.89	6958	1137	16261	6421	2229

parasites (34–44 h after invasion) for GDP-[2-^{3}H]Man labelling, and 10^9 parasites (34–44 h after invasion) for UDP-[6-^{3}H]GlcNAc labelling. Glycolipids were extracted with chloroform/methanol/water. Glycolipid extracts from parasites labelled *in vivo*, parasite lysates and membranes were analysed in parallel by TLC (Figure 1). To investigate the formation of non-mannosylated glycolipids, parasite lysates or membranes were labelled with UDP-[6-^{3}H]GlcNAc in the presence of 5 mM EDTA, 1 mM ATP and 1 mM CoA but without the addition of GDP-Man (Figure 1, lanes 1 and 2). These incubation conditions led to the formation of four major peaks, one of which was not a GPI (Pf j; Table 1). Two of these four peaks were identified as glucosamine-PtdIns (GlcN-PtdIns, Pf ζ) and GlcN-phosphatidyl(acyl)inositol (GlcN-acyl-PtdIns, Pf θ) respectively by their migration on TLC (in comparison with the well-characterized [^{3}H]glucosamine-labelled GPIs), sensitivity towards GPI-specific treatments (Table 1, and results not shown) and characterization of their core glycans by high-pH anion-exchange chromatography (Figures 2F and 2H). The fourth glycolipid was identified as GlcNAc-phosphatidylinositol (GlcNAc-PtdIns; Pf ζ') on the basis of its sensitivity to treatment with PtdIns-specific phospholipase C and its insensitivity to GPI-specific phospholipase D and deamination with nitrous acid (Table 1). Analysis of the UDP-[6-^{3}H]GlcNAc-labelled glycolipids synthesized by parasite lysates (Figure 1, lane 3) or membrane preparations (Figure 1, lane 4) supplemented with 1 mM GDP-Man, 1 mM CoA and 1 mM ATP showed significant differences in the incorporation of radioactivity into glycolipids and the labelling efficiency of single glycolipids. The radioactivity in glycolipids labelled in parasite membrane preparations was approx. 30–40 % less (assessed by scintillation counting of aliquots of the glycolipid extracts) when compared with parasite lysates prepared from the same number of parasites. UDP-[6-^{3}H]GlcNAc labelling of parasite lysates (Figure 1, lane 3) yielded a set of glycolipids resembling the GPI pattern found in metabolically labelled parasites. The glycolipids corresponding to the GPIs GlcN-PtdIns (Pf ζ), GlcNAc-PtdIns (Pf ζ'), GlcN-acyl-PtdIns (Pf θ), Man_4-GlcN-acyl-PtdIns (Pf γ), ethanolamine-phosphate-Man_3-GlcN-acyl-PtdIns (Pf β) and ethanolamine-phosphate-Man_4-GlcN-acyl-PtdIns (Pf α) were labelled whereas Man_2-GlcN-acyl-PtdIns (Pf ϵ) and Man_3-GlcN-acyl-PtdIns (Pf δ) were labelled only faintly. Man-GlcN-acyl-PtdIns (Pf η) was not detected. All GPIs (except one) found in metabolically labelled parasites were synthesized by parasite lysates. They were therefore suitable for the analysis of GPI synthesis. The difference between using lysates and using membranes lies in the presence of undefined amounts of soluble components in parasite lysates that are washed away during the preparation of membranes. The use of membrane preparations led to the labelling of GlcN-PtdIns, GlcNAc-PtdIns and GlcN-acyl-PtdIns. However, the mannosylated GPIs Man_2-GlcN-acyl-PtdIns, Man_3-GlcN-acyl-PtdIns and ethanolamine-phosphate-Man_4-GlcN-acyl-PtdIns (Pf α) were labelled only inefficiently and the other GPIs were almost undetectable. Nevertheless, the labelling of the GPI-anchor precursor Pf α in membrane preparations indicated that the membranes in principle are capable of synthesizing the complete set of malarial GPIs. The differences in labelling patterns between parasite lysates and membranes, especially the inefficient labelling of mannosylated GPIs by membranes, implies the loss of a soluble cofactor necessary for the efficient synthesis of mannosylated GPIs during the preparation of membranes.

GDP-[2-^{3}H]Man-labelled glycolipids (Pf α, Pf β, Pf γ, Pf δ, Pf ϵ and Pf η) synthesized by parasite lysates (Figure 1, lane 5) or membranes (Figure 1, lane 6) co-migrate with metabolically labelled GPIs (Figure 1, lane 6) that are known to contain mannose [28]. These glycolipids were identified as GPIs by enzymic and chemical treatments ([17] and results not shown). The spectrum of GPIs labelled in the presence GDP-[2-^{3}H]Man is identical for parasite lysates and membranes but the incorporation of radioactivity into glycolipids is up to 3-fold higher when using membranes. This shows that the transfer of mannosyl residues to GPI biosynthesis intermediates is efficient in the cell-free systems in the presence of 1 mM ATP, 1 mM

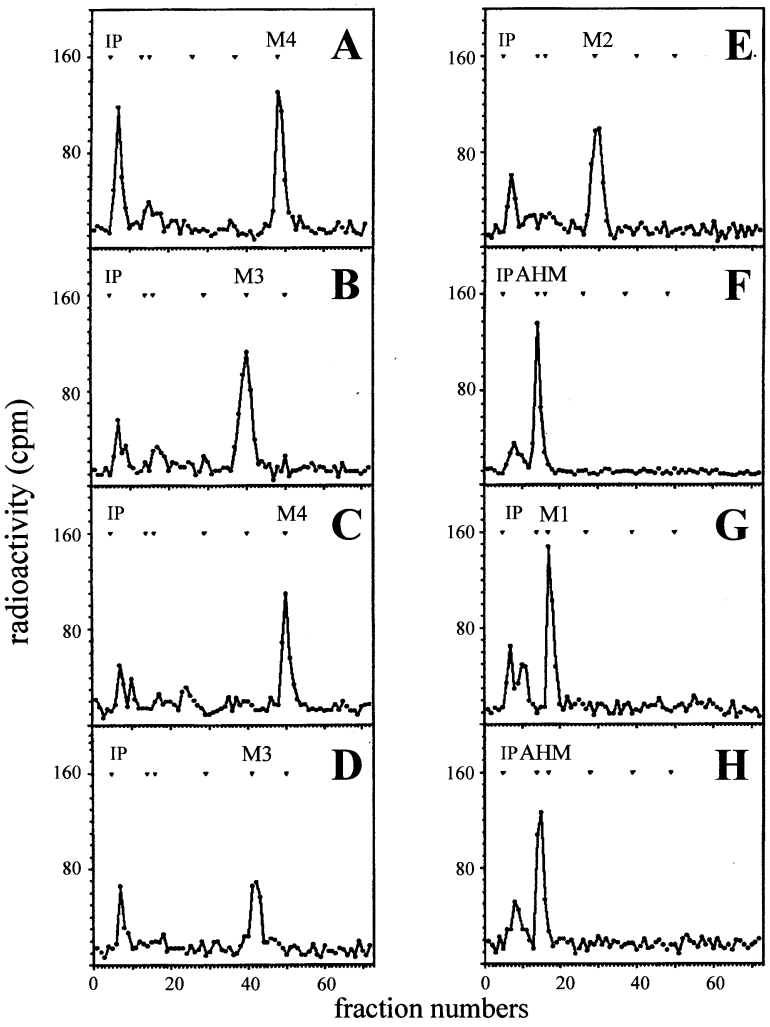

Figure 2 High-pH anion-exchange chromatography analysis of GPI core glycans

Neutral core glycans were generated by dephosphorylation, deamination and reduction of TLC-purified GPIs labelled by parasite lysates with tritiated GDP-Man [(**A**) Pf α; (**B**) Pf β; (**C**) Pf γ; (**D**) Pf δ; (**E**) Pf ϵ; (**G**) Pf η] or UDP-GlcNAc [(**F**) Pf ζ; (**H**) Pf θ]. Aliquots (1200–1500 c.p.m.) of each sample were analysed by high-pH anion-exchange chromatography (Dionex). The standards indicated at the top of each profile correspond to the elution positions of standard neutral core glycans. Abbreviations: AHM, anhydromannitol; M1, Man_1-anhydromannitol; M2, Man_2-anhydromannitol; M3, Man_3-anhydromannitol; M4, Man_4-anhydromannitol. Abbreviation: IP, injection peak.

CoA, 1 mM GTP and 1 mM UDP-GlcNAc. The core glycans of TLC-purified GDP-[2-^{3}H]Man (Pf α, Pf β, Pf γ, Pf δ, Pf ϵ and Pf η)-labelled or UDP-[6-^{3}H]GlcNAc (Pf ζ and Pf θ)-labelled glycolipids from parasite lysates were analysed by high-pH anion-exchange chromatography (Dionex Corp.). These analyses showed that Pf α and Pf γ contain a Man_4-GlcN core (Figures 2A and 2C), that Pf β and Pf δ contain a Man_3-GlcN core (Figures 2B and 2D), that Pf ϵ contains a Man_2-GlcN core (Figure 2E) and that Pf η contains a Man-GlcN core (Figure 2G), as described previously for their metabolically labelled counterparts [28]. In addition, Pf ζ and Pf θ have a non-mannosylated glucosamine residue (Figures 2F and 2H).

Thus, on the basis of the ability of cell-free systems to synthesize the same spectrum of GPIs as that found in metabolically labelled asexual stages of the parasite, investigations on the biosynthesis of GPIs in malarial parasites can make use of the cell-free incubation systems established from parasite lysates and membranes.

Synthesis of inositol acylated GPIs is stimulated by CoA

On the basis of the reports from other systems, especially trypanosomes, mammalian cells and *Leishmania* (reviewed in [2–8]), we supplemented our cell-free incubation systems with components known to be involved in GPI biosynthesis. To determine the requirements for efficient GPI biosynthesis by *P. falciparum*, we incubated membrane preparations in the presence of 1 mM ATP, 1 mM CoA or 1 mM GTP and either 1 mM UDP-GlcNAc or 1 mM GDP-Man. Furthermore, incubations with or without the addition of all these supplements were performed. Labelling was initiated by the addition of the corresponding tritiated nucleotide sugar (UDP-GlcNAc/GDP-[2-^{3}H]Man or GDP-Man/UDP-[6-^{3}H]GlcNAc). Subsequently, glycolipids were extracted and analysed by TLC.

In the presence of all supplements, labelling of mannosylated GPIs occurred with GDP-[2-^{3}H]Man (Figure 3A, lane 1). Without the addition of supplements, the yield of labelled GPIs was

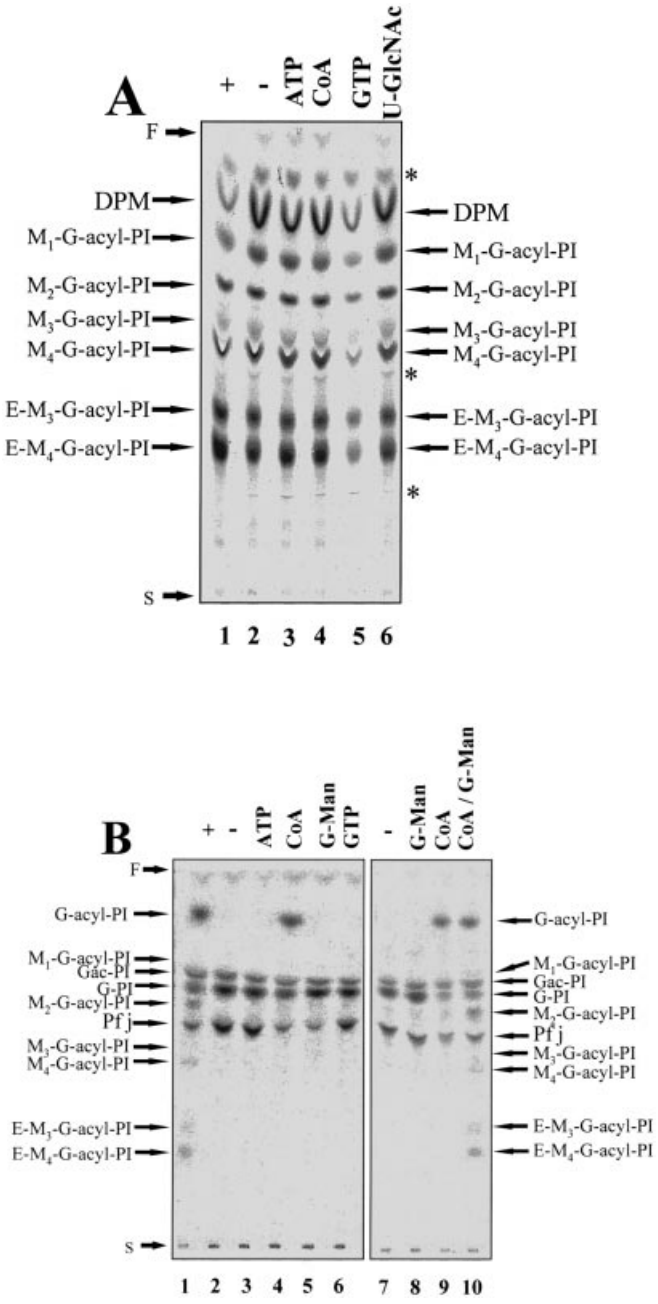

Figure 3 Effect of different supplements on GPI synthesis in parasite membrane preparations

Parasite membrane preparations were labelled with GDP-[2-^{3}H]Man (**A**) or UDP-[6-^{3}H]GlcNAc (**B**) in the presence of different supplements. Glycolipids were extracted and analysed by TLC. Radioactivity was detected with a BAS-1000 Bio-Imaging Analyser (Fuji Film). Glycolipids were designated as listed in the legend to Figure 1. Abbreviations: +, incubation mix contained CoA, ATP, GTP and the corresponding nucleotide sugar; —, incubation mix contained no exogenous supplements; G-Man, GDP-Man; PI, PtdIns; G, GlcN; Gac, GlcNAc; M, mannose; E, ethanolamine phosphate; S, migration start; F, solvent front. Pf j was identified as a non-GPI glycolipid.

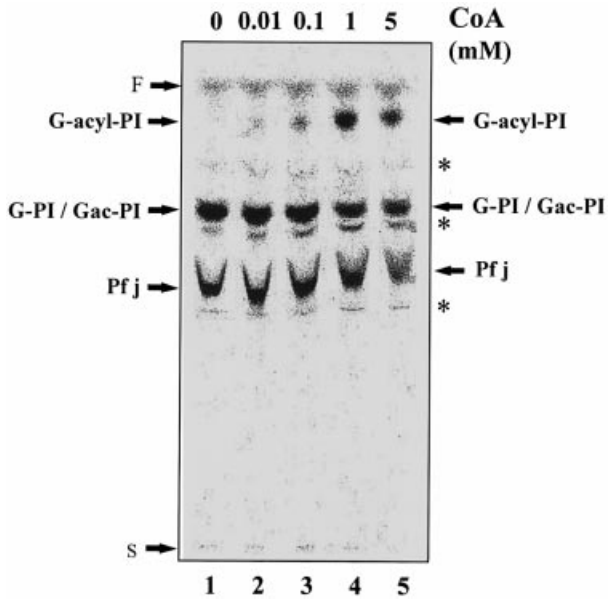

Figure 4 Effects of CoA on the formation of GlcN-acyl-PtdIns

Parasite membrane preparations were labelled with UDP-[6-^{3}H]GlcNAc in the presence of various concentrations of CoA. Glycolipids were extracted and analysed by TLC. The incorporation of radioactivity into individual glycolipids was determined by the peak integration software (TINA 2.0) of the Bio-Imager. Abbreviations: PI, PtdIns; G, glucosamine; *, uncharacterized glycolipid; S, migration start; F, solvent front. Pf j was identified as a non-GPI glycolipid.

approx. 25% higher than in the incubation containing all supplements. The addition of 1 mM ATP, CoA or UDP-GlcNAc separately had no effect on the labelling efficiency of malarial GPIs compared with the control without any supplement. Furthermore, the addition of 1 mM ATP (Figure 3A, lane 3), CoA (Figure 3A, lane 4) or UDP-GlcNAc (Figure 3A, lane 6) had no effect on the spectrum of GPIs synthesized. Supplementing parasite membranes with 1 mM GTP resulted in a marked decrease (approx. 79%) in the incorporation of radioactive mannose into the GPIs (Figure 3A, lane 5). The decreased incorporation of radioactivity into GPIs in the presence of all supplements compared with the control without the addition of supplements was probably caused by an inhibitory effect of GTP. We speculate that the effect of GTP is due to a competitive inhibition of the binding of GDP-Man to the dolichol-phosphate-mannose synthase.

To test the effects of the various supplements on the synthesis of the non-mannosylated GPIs, membrane preparations were labelled with UDP-[6-^{3}H]GlcNAc with (Figure 3B, lane 1) or without (Figure 3B, lane 2) the non-radioactive supplements present. Addition of the supplements led to a decrease in the radioactivity found in the GPIs by approx. 30% as determined by liquid-scintillation counting of aliquots of the glycolipid extracts. Furthermore, the spectrum of labelled glycolipids was different. Only in the presence of the supplements were GlcN-acyl-PtdIns and the mannosylated GPIs Pf α and Pf γ (both containing a Man_4 core glycan) detected in significant amounts; only marginal amounts of Pf β were detected (Figure 3B, lane 1). The addition of 1 mM ATP (Figure 3B, lane 3) or 1 mM GTP (Figure 3B, lane 6) to incubations had no effect on the amounts of radioactivity incorporated into GPIs and led to the labelling of only GlcN-PtdIns and GlcNAc-PtdIns. The addition of 1 mM GDP-Man to the incubation mix led to a decrease in the incorporation of glucosamine from UDP-[6-^{3}H]GlcNAc into GPIs by 50–60%. The only two GPIs labelled under these conditions were GlcN-PtdIns and GlcNAc-PtdIns (Figure 3B, lane 5). In addition, the non-GPI lipid Pf j was labelled. These

results imply that the acylation of the inositol ring, resulting in the formation of GlcN-acyl-PtdIns, is a prerequisite for the transfer of the first mannose residue in GPI biosynthesis in *P. falciparum*. Significant amounts of GlcN-acyl-PtdIns were detected only in the presence of 1 mM CoA (Figure 3B, lane 4). If membrane preparations were labelled with UDP-[6-^{3}H]GlcNAc in the presence of 1 mM CoA and 10 μM GDP-Man, the GPI-anchor precursors Pf α and Pf β were synthesized (Figure 3B, lane 10), showing that these two supplements were sufficient for synthesis of the complete set of GPIs by the plasmodial cell-free system. These results show that CoA is either involved in or stimulates the transfer of the acyl residue to the inositol. The addition of 10 μM CoA to membranes labelled with UDP-[6-^{3}H]GlcNAc led to a marginal stimulation of the synthesis of GlcN-acyl-PtdIns (Figure 4, lane 2). Only if the membrane preparation was supplemented with 100 μM (Figure 4, lane 3), 1 mM (Figure 4, lane 4) or 5 mM CoA (Figure 4, lane 5) was significant synthesis of GlcN-acyl-PtdIns observed, compared with the control without the addition of CoA (Figure 4, lane 1). The high concentrations of CoA needed to stimulate inositol acylation point to an indirect effect of CoA on inositol acylation in GPIs or the presence of a minor contaminating component (such as acyl-CoA) present in the commercial CoA preparation used, which might be the donor for the acyl chain at the inositol.

Myristoyl-CoA is the preferred donor for plasmodial inositol acylation in GPIs

To check for the direct involvement of acyl-CoA as the donor for inositol acylation in *P. falciparum*, membrane preparations were labelled with UDP-[6-^{3}H]GlcNAc in the presence of 100 μM acyl-CoA of various acyl chain lengths. Glycolipids were extracted by organic solvents and analysed by TLC. An increasing efficiency of acyl transfer to the inositol ring was shown for $C_{10:0}$ acyl-CoA (Figure 5, lane 5), $C_{14:1}$ acyl-CoA (Figure 5, lane 3), $C_{16:0}$ acyl-CoA (Figure 5, lane 2) and $C_{14:0}$ acyl-CoA (Figure 5, lane 4), respectively. $C_{22:0}$ was not transferred to inositol from $C_{22:0}$ acyl-CoA (Figure 5, lane 1). The transfer of acyl chains of different chain lengths to the inositol ring was demonstrated by the slightly different migration behaviours of the resulting GlcN-acyl-PtdIns species. In contrast, the migration position of GlcN-PtdIns was not affected by the addition of different acyl-CoAs. Labelling of GlcN-acyl-PtdIns was most efficient with $C_{14:0}$ acyl-CoA and $C_{16:0}$ acyl-CoA; $C_{16:0}$ acyl-CoA was about half as efficient a donor as $C_{14:0}$ acyl-CoA on the basis of comparison of peak intensities with the integration software (TINA 2.0) of the Bio-Imager. These results point to a preference of the GlcN-PtdIns/acyl-transferase of *P. falciparum* to transfer $C_{14:0}$ or $C_{16:0}$ acyl chains from acyl-CoA. In addition, metabolically labelled GlcN-acyl-PtdIns co-migrated with GlcN-acyl-PtdIns synthesized by parasite membranes in the presence of $C_{14:0}$ acyl-CoA.

Synthesis of mannosylated GPIs is affected by the concentration of GDP-Man

The presence of 1 mM GDP-Man led to a decrease in the incorporation of radioactivity into GPIs (Figure 3B, lane 5). By using parasite membrane preparations, we investigated the effect of different concentrations of GDP-Man added together with 1 mM ATP and 1 mM CoA. UDP-[6-^{3}H]GlcNAc-labelled glycolipids were extracted and analysed by TLC. The addition of increasing concentrations (100 nM to 10 μM) of GDP-Man resulted in a decrease in the radioactivity found in the glycolipids from approx. 114 % to 77 % compared with the control without

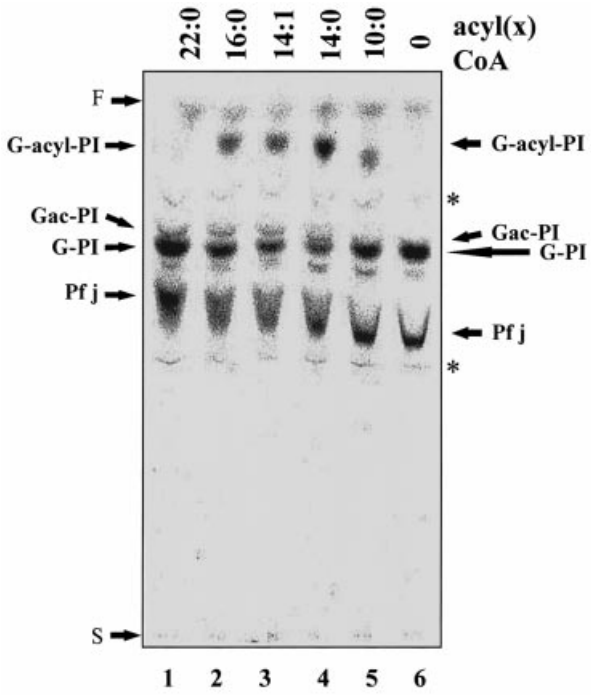

Figure 5 Effect of acyl-CoA chain length on the formation of GlcN-acyl-PtdIns (Pf θ)

Parasite membrane preparations were labelled with UDP-[6-^{3}H]GlcNAc in the presence of 100 μM acyl-CoA of different acyl chain lengths as indicated at the top (n:0 represents $C_{n:0}$). Pf θ indicates the migration position of Pf θ found in metabolically labelled parasites. The incorporation of radioactivity into individual glycolipids was determined by the peak integration software (TINA 2.0) of the Bio-Imager. Abbreviations: PI, PtdIns; G, glucosamine; Gac, GlcNAc; *, uncharacterized glycolipid; S, migration start; F, solvent front. Pf j was identified as a non-GPI glycolipid.

GDP-Man addition. Adding less than 100 nM GDP-Man to the incubation mix led to the incorporation of the same amount of radioactivity as that found in the control incubation without GDP-Man. The incorporation of radioactivity into glycolipids was measured by scintillation counting of aliquots of the individual samples.

In addition to differences in the incorporation of radioactivity into GPIs, the spectrum of glycolipids revealed in the TLC analysis changed (Figure 6). In the presence of 10–1 μM GDP-Man Pf α (ethanolamine-phosphate-Man$_4$-GlcN-acyl-PtdIns) and Pf γ (Man$_4$-GlcN-acyl-PtdIns) were the only mannosylated GPIs that were labelled efficiently (Figure 6, lanes 2 and 3). These two GPIs carry a trimannosyl core glycan modified by a fourth α1,2-Man residue. In the presence of 300–100 nM GDP-Man (Figure 6, lanes 4 and 5), the overall incorporation into mannosylated GPIs decreased but Pf β (ethanolamine-phosphate-Man$_3$-GlcN-acyl-PtdIns) and Pf δ (Man$_3$-GlcN-acyl-PtdIns) were labelled more efficiently. With the addition of 30 nM GDP-Man to the membranes (Figure 6, lane 6), the synthesis of Pf α and Pf γ was almost abolished. In the presence of 10 nM GDP-Man (Figure 6, lane 7), the synthesis of mannosylated GPIs was identical with that in the control without the addition of GDP-Man (Figure 6, lane 8). These results show that the mannosylation reactions in membrane preparations are affected differently by the concentration of GDP-Man present and that the minimal concentration of GDP-Man necessary to mannosylate UDP-[6-^{3}H]GlcNAc-labelled GPIs is approx.

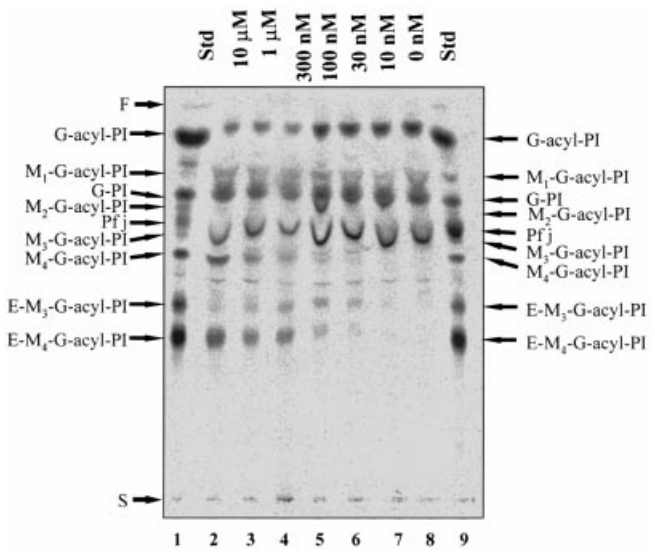

Figure 6 Effects of GDP-Man concentration on the synthesis of UDP-[6-^{3}H]GlcNAc-labelled GPIs

Parasite membrane preparations were labelled with UDP-[6-^{3}H]GlcNAc in the presence of increasing concentrations of GDP-Man, 1 mM ATP and 1 mM CoA. The migration positions of metabolically labelled plasmodial GPIs are indicated. The incorporation of radioactivity into individual glycolipids was determined by the peak integration software (TINA 2.0) of the Bio-Imager. The incorporation of radioactivity into glycolipid extracts was assessed by scintillation counting of aliquots of the different samples. Abbreviations: PI, PtdIns; G, glucosamine; M, mannose; E, ethanolamine phosphate; *, uncharacterized glycolipid; S, migration start; F, solvent front; Std, GPIs metabolically labelled with tritiated glucosamine. Pf j was identified as a non-GPI glycolipid.

30 nM in the cell-free system. In contrast, concentrations of 1 mM to 10 nM UDP-GlcNAc added to the incubation mix do not affect the labelling of GPIs by GDP-[2-^{3}H]Man (results not shown).

These results indicate that the concentration of GDP-Man present in the incubation has a tremendous effect on the mannosylation reactions on newly synthesized GPIs. This effect resembles the maturation-dependent formation of GPIs described for metabolically labelled parasites [29].

DISCUSSION

The general features of the biosynthesis of GPIs leading to the conserved GPI core structure are identical in all eukaryotes. Nevertheless, significant differences in the substrate and donor specificities of the enzymes involved in the early steps of the GPI biosynthetic pathway have been described with the use of cell-free incubation systems [10,11,25,27]. Detailed studies on GPI biosynthesis in *Plasmodium* might therefore lead to the identification of parasite-specific reactions of this pathway, which could be potential targets for the development of parasite-specific inhibitors.

The sequence of GPI biosynthesis in *Plasmodium* has been postulated on the basis of structural data and by analogy with other systems [28]. One interesting observation is that the malaria parasite *P. falciparum* synthesizes two GPI-anchor precursors (Pf α and Pf β), both of which were found on malarial proteins in a maturation-dependent manner [29]. Here we present results on cell-free incubation systems prepared from the asexual intra-erythrocytic stage of *P. falciparum*. A comparison of the labelling efficiencies of parasite lysates and parasite membrane preparations with GDP-[2-^{3}H]Man showed that membranes were approx. 3-fold more efficient than parasite lysates in incorporating mannose into GPIs. This might be due to the presence of intracellular GDP-Man in parasite lysates, which diluted the GDP-[2-^{3}H]Man used for labelling. The spectrum of glycolipids synthesized with GDP-[2-^{3}H]Man matched in parasite lysates and membrane preparations. All mannosylated GPIs described in metabolically labelled asexual stages of the parasite [28] could be efficiently labelled with lysates or membranes.

In the presence of UDP-[6-^{3}H]GlcNAc, parasite lysates and membranes incorporate less radioactivity into GPIs than with GDP-[2-^{3}H]Man. Furthermore, parasite lysates incorporated more UDP-[6-^{3}H]GlcNAc into GPIs than did membrane preparations; the labelling efficiencies of single glycolipids was different. In particular, GlcN-acyl-PtdIns, some of the mannosylated GPIs and the GPI-precursors Pf α (ethanolamine-phosphate-Man_4-GlcN-acyl-PtdIns) and Pf β (ethanolamine-phosphate-Man_3-GlcN-acyl-PtdIns) were labelled more efficiently with parasite lysates than with membranes. This leads us to suggest that parasite membrane preparations lack a cofactor necessary for the efficient synthesis of GPI-anchor precursors. However, we demonstrated that parasite lysates and membranes are capable of synthesizing the GPI-anchor precursor Pf α, the final precursor found in glycolipid extracts of metabolically labelled parasites. Therefore both cell-free incubation systems (lysates and membranes) were suitable for studies of the biosynthesis of glycolipids of *P. falciparum* and the identification of inhibitors of this pathway. We used membrane preparations for most experiments because they permit the study of concentration-dependent effects of exogenous supplements independently of intracellularly present soluble substances (which are removed during the preparation of membranes).

Further studies on the biosynthesis of GPIs dealt with the effects of the addition of various supplements to plasmodial membranes. A more detailed understanding of the requirements of parasite cell-free systems for the efficient synthesis of GPIs is a prerequisite for the development and testing of potential parasite-specific GPI biosynthesis inhibitors. Our studies show that supplementing parasite membranes with at least 100 μM CoA is a prerequisite for the efficient inositol acylation of GlcN-PtdIns. A stimulation of inositol acylation by CoA in the presence of GTP has been described for murine membrane preparations [16], with the use of 1 μM CoA or palmitoyl-CoA together with GTP. Interestingly, GTP alone had a stimulating effect on inositol acylation in murine membranes [16], whereas CoA alone did not lead to the formation of GlcN-acyl-PtdIns. These results were confirmed by Doerrler et al. [15], showing that the presence of CoA did not lead to the transfer of acyl chains to a synthetic short-chain GlcN-PtdIns in Chinese hamster ovary cell membranes. The stimulation of inositol acylation by acyl-CoA was found to be non-specific in murine membranes [16], whereas a specific transfer of fatty acids from acyl-CoA as a donor to a short-chain analogue of GlcN-PtdIns without the addition of GTP was shown in Chinese hamster ovary cells [15]. For *P. falciparum*, we have shown that the addition of GTP to parasite membranes did not support the formation of GlcN-acyl-PtdIns, whereas the addition of 100 μM (or more) CoA resulted in the transfer of an acyl chain to the inositol ring of endogenous GlcN-PtdIns. Furthermore, the present results demonstrate clearly an acyl-chain-length-dependent transfer of fatty acids from acyl-CoA to plasmodial GPIs. Taken together, our findings point to myristic acid as being the preferred fatty acid on the inositol ring of malarial GPIs. These results are also supported by structural results on the GPI anchors of the malarial merozoite proteins MSP-1 and MSP-2 [31], showing that the inositol ring of the GPI

anchors of these two proteins is preferentially substituted with myristic acid. Myristoylation of the inositol ring is a unique structural feature of malarial GPIs. The preference for myristic acid could make the plasmodial GlcN-PtdIns acyltransferase a target for the development of parasite-specific inhibitors. Inhibitors able to block inositol acylation would interfere with the synthesis of GPIs of *Plasmodium*, because inositol acylation is a prerequisite for subsequent mannosylation reactions as indicated by the absence of mannosylated GPIs in membrane preparations impaired in the formation of GlcN-acyl-PtdIns. This is similar to the situation found in yeast cell [12], rodent cell [32] and HeLa cell [11] membranes, but differs from findings with *T. brucei* [10,33].

The inhibitory effects of high concentrations of GDP-Man added to plasmodial cell-free systems lead us to suggest that GDP-Man affects the cell-free system by depleting a necessary component or by the direct regulation of GPI-mannosyltransferases. It can be speculated that the vast excess of GDP-Man added to the cell-free system might affect the formation of dolichol-phosphate-mannose, the ultimate mannosyl donor in GPI biosynthesis. A direct effect of GDP-Man concentrations on GPI-mannosyltransferases has not yet been investigated, probably because these enzymes are not available for enzymic and biochemical studies, although some of them have been cloned from mammalian cells and yeast (reviewed in [2,8]). We speculate that a maturation-dependent variation in the intracellular concentration of GDP-Man might be the regulatory element for the difference in the synthesis of the two GPI-anchor precursors of *P. falciparum* observed during maturation of the asexual, intraerythrocytic stage of *P. falciparum* [29].

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REFERENCES

1 Schofield, L. and Tachado, S. D. (1996) Immunol. Cell Biol. **74**, 555–563
2 Stevens, V. L. (1993) Biochem. J. **310**, 361–370
3 Field, M. C. and Menon, A. K. (1994) in Lipid Modification of Proteins (Schlesinger, M. J., ed.), pp. 83–134, CRC Press, Boca Raton, FL
4 Ferguson, M. A. J., Brimacombe, J. S., Cottaz, S., Field, R. A., Güther, L. S., Homans, S. W., McConville, M. J., Mehlert, A., Milne, K. G. and Ralton, J. E. (1994) Parasitology **108**, 45–54
5 McConville, M. J. (1996) in Molecular Biology of Parasitic Protozoa (Smith, D. F. and Parsons, M., eds.), pp. 205–228, Oxford University Press, Oxford
6 Gerold, P., Eckert, V. and Schwarz, R. T. (1996) Trends Glycosci. Glycotechnol. **8**, 265–277
7 Nosjean, O., Briolay, A. and Roux, B. (1997) Biochim. Biophys. Acta **1331**, 153–186
8 Kinoshita, T., Ohishi, K. and Takeda, J. (1997) J. Biochem. (Tokyo) **122**, 251–257
9 Sharma, D. K., Smith, T. K., Weller, C. T., Crossman, A., Brimacombe, J. S. and Ferguson, M. A. J. (1999) Glycobiology **9**, 415–422
10 Smith, T. K., Milne, F., Sharma, D. K., Crossman, A., Brimacombe, J. S. and Ferguson, M. A. J. (1997) Biochem. J. **326**, 393–400
11 Smith, T. K., Sharma, D. K., Crossman, A., Dix, A., Brimacombe, J. S. and Ferguson, M. A. J. (1997) EMBO J. **16**, 6667–6675
12 Costello, L. C. and Orlean, P. (1992) J. Biol. Chem. **267**, 8599–8603
13 Menon, A. K., Mayor, S. and Schwarz, R. T. (1990) EMBO J. **9**, 4249–4258
14 Field, M. C., Menon, A. K. and Cross, G. A. M. (1991) EMBO J. **10**, 2731–2740
15 Doerrler, W. T., Ye, J., Falck, J. R. and Lehrmann, M. A. (1996) J. Biol. Chem. **271**, 27031–27038
16 Stevens, V. L. and Zhang, H. (1994) J. Biol. Chem. **269**, 31397–31403
17 Gerold, P., Schuppert, A. and Schwarz, R. T. (1992) Trans. Biochem. Soc. **20**, 297S
18 Puoti, A., Desponds, C., Fankhauser, C. and Conzelmann, A. (1991) J. Biol. Chem. **266**, 21051–21056
19 Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A. and Benachour, A. (1998) Glycobiology **8**, 761–770
20 Sütterlin, C., Escribano, M. V., Gerold, P., Maeda, Y., Mazon, M. J., Kinoshita, T., Schwarz, R. T. and Riezman, H. (1998) Biochem J. **332**, 153–159
21 Sütterlin, C., Horvath, A., Gerold, P., Schwarz, R. T., Wang, Y., Dreyfuss, M. and Riezman, H. (1997) EMBO J. **16**, 6374–6383
22 Ralton, J. E., Milne, K. G., Guther, M. L., Field, R. A. and Ferguson, M. A. (1993) J. Biol. Chem. **268**, 24183–24189
23 Güther, M. L., Masterson, W. J. and Ferguson, M. A. (1994) J. Biol. Chem. **269**, 18694–18701
24 Heise, N., Raper, J., Buxbaum, L. U., Peranovich, T. M. and de Almeida, M. L. (1996) J. Biol. Chem. **271**, 16877–16887
25 Masterson, W. J., Doering, T. L., Hart, G. W. and Englund, P. T. (1989) Cell **56**, 793–800
26 Menon, A. K., Schwarz, R. T., Mayor, S. and Cross, G. A. M. (1990) J. Biol. Chem. **265**, 9033–9042
27 Doering, T. L., Masterson, W. J., Englund, P. T. and Hart, G. W. (1989) J. Biol. Chem. **264**, 11168–11173
28 Gerold, P., Schuppert, A. and Schwarz, R. T. (1994) J. Biol. Chem. **269**, 2597–2606
29 Schmidt, A., Schwarz, R. T. and Gerold, P. (1998) Exp. Parasitol. **88**, 95–102
30 Gowda, D. C., Gupta, P. and Davidson, E. A. (1997) J. Biol. Chem. **272**, 6428–6439
31 Gerold, P., Schofield, L., Blackman, M. J., Holder, A. A. and Schwarz, R. T. (1996) Mol. Biochem. Parasitol. **75**, 131–143
32 Urakaze, M., Kamitani, T., DeGaspari, R., Sugiyama, E., Chang, H.-M., Warren, C. D. and Yeh, E. T. H. (1992) J. Biol. Chem. **267**, 6459–6462
33 Smith, T. K., Cottaz, S., Brimacombe, J. S. and Ferguson, M. A. J. (1996) J. Biol. Chem. **271**, 6476–6482

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