Glycosylphosphatidylinositols of *Plasmodium chabaudi chabaudi*: a basis for the study of malarial glycolipid toxins in a rodent model

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Free and protein-bound glycosylphosphatidylinositols (GPIs) of the blood stages of the rodent malarial parasite *Plasmodium chabaudi chabaudi* AS were identified and characterized. TLC analysis of material extracted by organic solvents from metabolically labelled parasites revealed a distinct set of glycolipids. These glycolipids were identified as GPIs by specific chemical and enzymic treatments and by structural analysis of their glycan and hydrophobic parts. These analyses revealed that *P. c. chabaudi* AS synthesizes a set of GPI-biosynthesis intermediates

and two potential GPI-anchor precursors exhibiting the following structures: ethanolamine-phosphate [(α 1-2)mannose]mannose(α 1-2)mannose(α 1-6)mannose(α 1-4)glucosamine-(acyl)inositol-phosphate-diacylglycerol (P. ch. α) and ethanolamine-phosphate-mannose(α 1-2)mannose(α 1-6)mannose(α 1-4)glucosamine-(acyl)inositol-phosphate-diacylglycerol (P. ch. β). One of these GPI-anchor precursors (P. ch. α) possesses the same carbohydrate structure as the GPI membrane anchor of merozoite surface protein-1 from *P. c. chabaudi* AS.

INTRODUCTION

Glycosylphosphatidylinositols (GPIs) of *Plasmodium falciparum* have been suggested to be a new type of malarial toxin. *In vitro* studies showed that malarial GPIs are involved in the induction of release of tumour necrosis factor α and interleukin 1 α and the activation of inducible NO synthase in host macrophages [1–4]. In rodent model systems the increase in cytokine release by host cells during the course of a blood-stage malarial infection has been implicated as the major cause of death [5,6]. The potency of *P. falciparum* GPIs to induce cytokine release and thus cause severe pathology has been shown in a heterologous *in vivo* system. Mice injected with GPIs of the human malarial parasite *P. falciparum* subsequently showed pathological features similar to those described for severe human malaria [1,7,8]. The study of the pathological effects of malarial glycolipid toxins in infected human hosts or *in vitro* using cell-culture systems has a number of limitations. *Plasmodium chabaudi chabaudi* infection in laboratory mice is a rodent malaria model widely used for *in vivo* studies related to infection of humans by *P. falciparum*. Therefore structural analysis of *P. c. chabaudi* GPIs may facilitate the study of GPI function *in vivo*.

The first complete GPI structures were solved for the GPI anchors of *Trypanosoma brucei brucei* variant surface glycoprotein [9] and the rat brain Thy-1 [10]. Since then many more GPIs of mammalian and protozoan origin have been described (for reviews see [11–13]). GPI structures show several evolutionarily conserved features: glycerol- or ceramide-based hydrophobic domains, inositol phosphate, a non-acetylated glucosamine linked to a trimannosyl core glycan, and an ethanolamine phosphate at the non-reducing end of the glycan chain. The amino group of the ethanolamine is linked to the C-terminal amino acid of a GPI-anchored protein [14]. GPI biosynthesis follows a conserved pathway (reviewed in [15]), but variation is observed, mainly with regard to acylation of the inositol ring and the addition of further components to the highly conserved core structure (for reviews see [11–13]). The GPI anchor is synthesized

as a precursor glycolipid and transferred *en bloc* to the nascent protein chains in the endoplasmic reticulum by replacing the C-terminal amino acids in a transamidase-like reaction (for a recent review see [16]).

Detailed structural analysis of the GPIs synthesized by blood-stage *P. c. chabaudi* parasites is a prerequisite for studying the pathological effects of these glycolipids. Subsequent *in vivo* studies of the toxic effects of parasite GPIs in a rodent malaria model may provide a deeper insight into the relationship between malarial glycolipids and the host immune response. We thus laid the basis for these studies by identifying and characterizing free and protein-bound GPIs of *P. c. chabaudi* AS.

MATERIALS AND METHODS

Materials

D-[6-³H]Glucosamine hydrochloride (26.0 Ci/mmol) was purchased from NEN–DuPont. Phosphatidylinositol-specific phospholipase C (PI-PLC) of *Bacillus cereus* was from Boehringer-Mannheim. Jack-bean α -mannosidase and *Aspergillus saitoi* α 1-2-specific mannosidase were obtained from Oxford Glycosystems. Tunicamycin was purchased from Calbiochem. Ion-exchange resins were analytical grade and purchased from Bio-Rad. All sugar and phospholipid standards were obtained from Sigma or prepared by described procedures. All solvents were of analytical or HPLC grade.

Parasites and culture

The cloned line of *P. c. chabaudi* (strain AS) was obtained from the World Health Organisation Registry of Standard Strains of Malaria Parasites (Edinburgh, Scotland, U.K.).

Blood-stage parasites were maintained in CBA/Ca mice. Blood was harvested when the parasitaemia had reached 20–30 % and parasites were predominantly mature forms, as verified by

Abbreviations used: GPI, glycosylphosphatidylinositol; MSP, merozoite surface protein; HPAEC, high-pH anion-exchange chromatography; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PLD, GPI-specific phospholipase D.

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microscopic examination of Giemsa-stained blood smears [17]. Leucocytes were removed from the parasitized erythrocytes by passage through a CF11 column (Whatman).

Metabolic labelling

Parasitized erythrocytes were metabolically labelled essentially as described [18]. Briefly, parasites were cultured in glucose-free RPMI medium (Amimed, Muttentz, Switzerland) supplemented with 20 mM fructose, 50 mM Hepes, 21 mM NaHCO₃, 0.37 mM hypoxanthine, 0.1 mg/ml neomycin, 0.1 mM methionine, 2 mM glutamine and 11 mM glutathione medium containing 0.5% Albumax I (Life Technology) until most had developed into schizonts. Subsequently they were labelled in glucose-free RPMI medium (see above) with 200–250 µCi of [³H]glucosamine, [³H]ethanolamine or [³H]myristic acid per 10⁹ parasites in 10 ml of labelling medium. Tunicamycin (0.5 µg/ml) was added to the medium when indicated. All labelling experiments were carried out at 37 °C for 1–3 h.

Extraction and purification of glycolipids

Washed blood containing parasitized erythrocytes was subjected to saponin lysis to release parasites [19]. Glycolipids were extracted with chloroform/methanol (1:1, v/v) as described [20]. The organic solvent extracts were pooled and subjected to repeated Folch washing, dried and partitioned between water and water-saturated butan-1-ol [18]. The washed extracts were analysed by TLC on Si-60 plates (Merck, Darmstadt, Germany) using either chloroform/methanol/water (4:4:1, by vol.; solvent A) or chloroform/methanol/conc. acetic acid/water (25:15:4:2, by vol.; solvent B) as solvent [18].

Radioactivity was analysed using a BAS-1000 BioImager (Fuji). Single glycolipids were purified from TLC plates as described [18].

Characterization of glycolipids

Metabolically labelled glycolipids (TLC-purified or within the glycolipid mixture) were identified as GPIs by specific enzymic and chemical treatments including PI-PLC, GPI-specific phospholipase D (GPI-PLD) and HNO₂ deamination, as described previously [18,20]. The hydrophobic fragments were characterized by alkaline saponification [21] and TLC analysis of the hydrophobic fragments generated by HNO₂ deamination using solvent system A. Sensitivity of glycolipids to specific treatments was assessed by butanol/water phase separation of the reaction mixtures and TLC analysis of the organic phases [18].

Generation and analysis of hydrophilic fragments and neutral core glycans

Hydrophilic fragments were prepared from TLC-purified glycolipids by deamination with HNO₂ as described [22]. The released hydrophilic fragments were analysed on a Bio-Gel P4 size-exclusion column. Their elution position is given in glucose units, referring to partially hydrolysed dextran as internal standard [18].

Neutral core glycans were prepared from TLC-purified glycolipids by dephosphorylation, deamination and reduction as described [20]. Desalted neutral core glycans were analysed by TLC [23] on Si-60 HPTLC plates (Merck) using propan-1-ol/acetone/water (9:6:5, by vol.) as solvent [23] (solvent system C) or by high-pH anion-exchange chromatography (HPAEC) (Dionex Corp.). The HPAEC analysis was accomplished by gradient elution using a Carboapak[®] PA1 column (4 mm ×

250 mm). Partially hydrolysed dextran was added to the sample as internal standard [22]. Neutral core-glycan standards were prepared from radiolabelled GPIs of *T. b. brucei* and rat brain Thy-1.

The elution programme for HPAEC was 100% buffer A (0.1 M NaOH), 0% buffer B (0.1 M NaOH, 0.25 M sodium acetate) up to 6 min after injection, then an increase in buffer B to 30% over 36 min, at a flow rate of 1 ml/min.

Generation and analysis of the hydrophobic fragment of P. ch. α

About 7000 c.p.m. of the potential GPI-anchor precursor P. ch. α labelled with [³H]myristic acid was solubilized in 10 µl of methanol and treated with HNO₂ [18]. The hydrophobic fragment released was subjected to butanol/water phase separation as described [18], and the organic phase was analysed by TLC using solvent system A along with phosphatidylinositol standards derived from fatty acid-labelled P2 (glycolipid A) and P3 (glycolipid C) of *T. b. brucei* [24].

Purification of the GPI peptide and characterization of the glycan part released from the merozoite surface protein-1 (MSP-1)

MSP-1 was purified by immunoprecipitation using the monoclonal antibody NIMP23 [25] from detergent lysates of glucosamine-labelled parasites [21]. To remove non-covalently linked glycolipids, 4 vol. of methanol was added to 1 vol. of lysate and mixed. After the addition of 1 vol. of chloroform and 3 vol. of water, the suspension was mixed and centrifuged for 2 min at 14000 g at 4 °C. The upper aqueous phase was removed and the lower phase was mixed with 3 vol. of methanol before centrifugation at 14000 g for 10 min at 4 °C. The precipitated protein was extracted an additional three times with 5 vol. of chloroform/methanol/water (10:10:3, by vol.). After the final extraction step, radioactivity in the last wash was less than 1% of that found in the GPI peptide (see below). Residual chloroform/methanol/water was removed by acetone precipitation, and the protein was then treated with Pronase [21]. The GPI peptide was purified from the peptide mixture on octyl-Sepharose [22]. The structure of the glycan core of the GPI peptide was investigated by generating neutral core glycans by dephosphorylation, deamination and reduction and subsequent analysis by HPAEC or TLC using solvent system C. The hydrophilic fragment generated by deamination was analysed on Bio-Gel P4 size-exclusion columns (see below).

Generation and analysis of the hydrophobic fragment of MSP-1

About 3500 c.p.m. of Pronase-generated MSP-1 GPI peptide labelled with [³H]myristic acid were treated with HNO₂ as described [18]. The hydrophobic fragment released (about 2500 c.p.m.) was subjected to butanol/water phase separation [18], and the organic phase was analysed by TLC using solvent system A along with phosphatidylinositol standards derived from fatty acid-labelled P2 (glycolipid A) and P3 (glycolipid C) of *T. b. brucei* [24].

Determination of location of the ethanolamine phosphate group

Glycolipid extracts from [³H]glucosamine-labelled parasite cultures were divided into two aliquots, dried in a Speed-vac and solubilized in Jack-bean α-mannosidase [18] buffer containing 0.5% Nonidet P40. Jack-bean α-mannosidase (10 units) was added to one aliquot and incubated at 37 °C, whereas the other aliquot was incubated without enzyme. After 24 h the incubation was stopped by heating the sample for 3 min at 100 °C. After

butanol/water phase partition, the organic phases were analysed by TLC using solvent system A [18].

Preparation of GPI standards from *T. b. brucei* and rat brain Thy-1

Standard GPIs from *T. b. brucei* were prepared as described previously [18]. Fatty acid-labelled GPI standards from *T. b. brucei* were prepared as described [24]. Rat brain Thy-1 GPI-anchor core glycan with the structure Man(α 1-2)Man(α 1-2)Man(α 1-6)Man(α 1-4)anhydromannitol was kindly provided by Dr. M. A. J. Ferguson.

RESULTS

Identification of GPIs synthesized by blood-stage forms of *P. c. chabaudi* AS

P. c. chabaudi AS was grown in CBA/Ca mice. Blood was harvested, and the leucocyte-depleted parasitized erythrocytes were labelled with [3 H]glucosamine. Glycolipids were extracted with organic solvents and purified by Folch washing and butanol/water phase separation. The resulting glycolipid extract was subjected to TLC analysis using solvent system A (Figure 1) or solvent system B (results not shown) along with *P. falciparum* GPIs of known structure. The comparison of [3 H]glucosamine-labelled glycolipids from *P. c. chabaudi* AS with the spectrum of

Figure 1 Comparison of [3 H]glucosamine-labelled glycolipids from *P. c. chabaudi* AS and *P. falciparum*

Blood-stage parasites were metabolically labelled with [3 H]glucosamine for 1–2 h. Glycolipids were extracted with chloroform/methanol/water and analysed by TLC using solvent system A (lane A). Glycolipid extracts containing *P. falciparum* GPIs were analysed in parallel (lane B). Radioactivity was measured using a BAS-1000 BioImaging analyser (Fuji). Eight glucosamine-labelled glycolipids from *P. c. chabaudi* were identified and designated P.ch. α , P.ch. β , P.ch. γ , P.ch. δ , P.ch. ϵ , P.ch. ζ , P.ch. η and P.ch. θ .

Scheme 1 Schematic drawing of a GPI and its cleavage sites

Cleavage sites of different chemical and enzymic treatments specific for GPIs are indicated by the arrows. PI-PLC cleaves only if the inositol is not substituted; HF, dephosphorylation by HF; EtN, ethanolamine; Man, mannose; GlcN, glucosamine.

Table 1 Characterization of metabolically labelled glycolipids from *P. c. chabaudi* AS

Glycolipids were extracted from [3 H]glucosamine-labelled blood-stage parasites and analysed by TLC. Single glycolipids were purified from TLC plates and subjected to enzymic and chemical treatments specific for GPIs. Cleavage was assessed by butanol/water phase partition, and radioactivity in the organic phases was analysed by scintillation counting. Control incubations (without treatment) were set as 100% recovery.

	Recovery (%)			
	PI-PLC	GPI-PLD	HNO ₂	NaOH
P.ch. α	102	15	57	3
P.ch. β	105	25	62	2
P.ch. γ	98	16	64	3
P.ch. δ	93	18	58	5
P.ch. ϵ	83	17	52	9
P.ch. ζ	8	12	47	4
P.ch. η	85	27	54	5
P.ch. θ	95	38	45	2

GPIs synthesized by intraerythrocytic stages of *P. falciparum* showed a qualitatively identical TLC pattern using solvent systems A and B respectively; however, GPIs of *P. c. chabaudi* AS migrated slightly more hydrophobically than the corresponding GPIs of *P. falciparum* (Figure 1, solvent system A). The *P. c. chabaudi* AS glycolipids are designated P.ch. α , P.ch. β , P.ch. γ , P.ch. δ , P.ch. ϵ , P.ch. ζ , P.ch. η and P.ch. θ . Parasite extracts containing glycolipids metabolically labelled with [3 H]ethanolamine were analysed in parallel. More than 99% of the radioactivity found in the [3 H]ethanolamine-labelled glycolipid extracts co-migrated with phosphatidylethanolamine and phosphatidylserine standards. In addition, two minor [3 H]ethanolamine-labelled peaks, co-migrating with glycolipids P.ch. α and P.ch. β , were found in this extract (results not shown).

To check for the presence of lipid-linked intermediates of the dolichol cycle, parasites were labelled with [3 H]glucosamine in the presence or absence of tunicamycin. After organic solvent extraction and Folch washing, the glycolipid extracts were analysed by TLC. No difference in the glycolipid pattern was observed for parasites labelled in the presence or absence of tunicamycin (results not shown). This implies that dolichol-cycle intermediates may only be present in amounts below the level of detection.

The *P. c. chabaudi* AS glycolipids were identified as GPIs by subjecting whole glycolipid extracts or TLC-purified glycolipids to different chemical and enzymic treatments specific for GPIs (Scheme 1). Efficiency of cleavage of the glycolipid extracts was

Figure 2 HPAEC analysis of GPI core glycans

Neutral core glycans were generated by dephosphorylation, deamination and reduction of metabolically labelled TLC-purified GPIs. Aliquots (1200–1500 c.p.m.) of each sample were analysed by HPAEC (Dionex). The standards indicated at the top of each profile correspond to the elution position of standard neutral core glycans. A, anhydromannitol; M1, Man₁-anhydromannitol; M2, Man₂-anhydromannitol; M3, Man₃-anhydromannitol; M4, Man₄-anhydromannitol.

assessed after butanol/water phase separation by analysing the butanol phase on TLC (results not shown). In addition, single purified glycolipids were subjected to these treatments and cleavage efficiency was assessed by butanol/water phase separation and determination of the radioactivity in the organic phase by scintillation counting (Table 1). All [³H]glucosamine-labelled glycolipids from *P. c. chabaudi* AS were shown to be sensitive to treatment with HNO₂ and GPI-PLD. All but one (P.ch. ζ) of these glycolipids were insensitive to PI-PLC, showing that these GPIs possess a modified inositol structure. As P.ch. ζ was found to be sensitive to PI-PLC, this implies that this glycolipid is a GPI without a modification at the inositol ring. The hydrophobic part of all *P. c. chabaudi* AS GPIs was shown to be sensitive to alkaline saponification specific for ester-linked fatty acids (Table 1). Therefore we concluded that *P. c. chabaudi* AS synthesizes acylglycerol-based GPIs carrying (except for

Figure 3 Characterization of the mannose linkages found in the core glycans of the potential GPI-anchor precursors

Neutral core glycans of the potential GPI-anchor precursors P.ch. α (lanes 1–3) and β (lanes 4–6) were treated with buffer alone (lanes 1 and 4), *A. saitoi* α1,2-mannosidase (lanes 2 and 5) or Jack-bean α-mannosidase (lanes 3 and 6). The probes were desalted on mix-bed ion-exchange columns and analysed by TLC using solvent system C. Radioactivity was measured using a BAS-1000 BioImaging analyser (Fuji). Man4, Man(α1-2)Man(α1-2)Man(α1-6)Man(α1-4)anhydromannitol; Man3, Man(α1-2)Man(α1-6)Man(α1-4)anhydromannitol; Man2, Man(α1-6)Man(α1-4)anhydromannitol; Man1, Man(α1-4)anhydromannitol; AHM, anhydromannitol; O, origin, F, front.

P.ch. ζ) an ester-linked modification at the inositol ring that renders them insensitive to PI-PLC treatment.

Characterization of the hydrophilic part of the free GPIs

To characterize the hydrophilic part of the *P. c. chabaudi* AS GPIs, TLC-purified [³H]glucosamine-labelled glycolipids were subjected to dephosphorylation, deamination and reduction to release the neutral glycan from each GPI. HPAEC analysis of the desalted neutral core glycans using neutral core glycans of known structure from *T. b. brucei* and rat brain Thy-1 as standards showed that the parasite synthesizes a set of differently mannosylated GPI core glycans (Figure 2). Two *P. c. chabaudi* AS GPIs (Figure 2; P.ch. ζ and θ) were shown to co-migrate with the anhydromannitol standard, and therefore corresponding to GPIs originally carrying glucosamine only. All other *P. c. chabaudi* AS GPIs possessed neutral core glycans mannosylated by one (Figure 2; P.ch. η), two (Figure 2; P.ch. ε), three (Figure 2; P.ch. β and P.ch. δ) or four (Figure 2; P.ch. α and P.ch. γ) α-linked mannose residues. The radioactivity eluted shortly after the injection in some analyses may be due to unspecific adsorbance of core glycans to unlabelled material which does not bind to the column. In addition to the highly specific HPAEC analysis, the type of linkage between the mannoses was confirmed by treatment with Jack-bean α-mannosidase. The mannoses found in the core glycans of *P. c. chabaudi* AS GPIs were shown to be completely sensitive to α-mannosidase, implying that all mannoses are in α-linkage (for P.ch. α and β see Figure 3). The linkage type of the terminal mannoses found in P.ch. α and β was further characterized. After treatment with *A. saitoi* α1,2-mannosidase the neutral core glycans from both GPIs co-migrated with the Man(α1-6)Man(α1-4)anhydromannitol standard on TLC using solvent system C (Figure 3).

Figure 4 Characterization of the phosphatidylinositol fragment of P.ch. α

The phosphatidylinositol fragment released from [^3H]myristic acid-labelled P.ch. α by HNO_2 deamination was analysed by TLC using solvent system A. PI, migration position of phosphatidylinositol; acyl-PI, migration position of dimyristoyl-phosphatidyl(palmitoyl)inositol; P.ch. α , migration position of P.ch. α ; O, origin; F, front.

The location of the ethanolamine phosphate found in all potential GPI-anchor precursors was established by characterizing the blocked mannose core structure [18]. The mixture of [^3H]glucosamine-labelled GPIs was subjected to Jack-bean α -mannosidase treatment. Analysis of the organic phases on TLC using solvent system A showed that α -mannosidase treatment leads to a decrease in radioactivity in P.ch. α and the other mannosylated GPIs. P.ch. β was the major mannosylated GPI found (results not shown). A large proportion of the radioactivity was found in the non-mannosylated GPIs P.ch. ζ and P.ch. θ . These data imply that the mannosylated GPIs are sensitive to α -mannosidase treatment and that the fourth mannose found in P.ch. α represents a side branch of the trimannosyl core glycan. These data suggest that the ethanolamine phosphate is linked to the third mannose of the core glycan in P.ch. α and P.ch. β , as has been described for other GPI-anchor precursors [11–13].

Characterization of the hydrophobic fragment of P.ch. α

The sensitivity of most GPIs of *P. c. chabaudi* AS to treatment with PI-PLC, their chromatographic behaviour on TLC and their sensitivity to alkaline treatment imply that this rodent malarial parasite possesses GPIs with an inositol ring modified by an ester-linked fatty acid (except P.ch. ζ) and a diacylglycerol-based membrane anchor. The hydrophobic fragment of TLC-purified P.ch. α was further characterized by analysing the phosphatidylinositol moiety generated by HNO_2 deamination on TLC using solvent system A. This fragment released from P.ch. α migrated far more hydrophobically than the dimyristoyl-phosphatidylinositol standard generated from [^3H]myristic acid-labelled P2 (glycolipid A) of *T. b. brucei* and a commercially available phosphatidylinositol standard (Figure 4). The hydro-

Table 2 Identification of the GPI anchor on MSP-1

MSP-1 was purified from lysates of [^3H]glucosamine-labelled blood-stage parasites by immunoprecipitation. The GPI peptide, generated by Pronase treatment, was purified by phase separation between water-saturated butan-1-ol and water, and then characterized by different chemical and enzymic GPI-specific treatments. Cleavage was determined by liquid-scintillation counting after phase separation between water-saturated butan-1-ol and water.

	Recovery (%)					
	HNO_2	NaCl	NaOH	GPI-PLD	PI-PLC	Control
Organic phase	47.9	98.1	3.2	61.2	94.5	96.5
Aqueous phase	52.1	1.9	96.8	38.8	5.5	3.5

phobic fragment of P.ch. α migrated in the same region as, but slightly more hydrophobically than, the dimyristoyl-phosphatidyl(palmitoyl)inositol standard generated from [^3H]myristic acid-labelled P3 (glycolipid C) of *T. b. brucei*. These data confirm the results obtained by treatment with PI-PLC, the chromatographic behaviour on TLC, and its sensitivity to alkaline treatment and suggest that *P. c. chabaudi* GPI-anchor precursor P.ch. α possesses a hydrophobic acylated phosphatidylinositol.

Structural analysis of the MSP-1 GPI anchor

The GPI-anchor peptide of *P. c. chabaudi* AS MSP-1 was purified from metabolically labelled parasites by immunoprecipitation, Pronase treatment and subsequent purification of the hydrophobic GPI peptide by hydrophobic interaction chromatography on octyl-Sepharose. The purified GPI-anchor peptide labelled with [^3H]glucosamine was characterized by chemical and enzymic treatments specific for GPIs (Table 2). The GPI anchor of MSP-1 was shown to be insensitive to PI-PLC treatment but was identified as a GPI anchor by its sensitivity to treatments with GPI-PLD and HNO_2 (Table 2). The GPI anchor of the MSP-1 therefore shows the same susceptibility to GPI-specific treatments as most non-protein-bound malarial GPIs. This implies that the protein-bound GPI anchor also carries a modified inositol ring. Detailed characterization of the [^3H]glucosamine-labelled hydrophilic part of the protein-bound GPI by analysing the neutral core glycan by HPAEC and TLC showed the presence of the evolutionarily conserved trimannosyl core glycan modified by an additional α -linked mannose residue (Figure 5A). The presence of an additional mannose was confirmed by analysing the hydrophilic fragment of the MSP-1 GPI peptide prepared by HNO_2 deamination on a Bio-Gel P4 size-exclusion chromatography column. This fragment was eluted at 9.3 glucose units from a Bio-Gel P4 size-exclusion column (Figure 5B). The corresponding fragment from the free GPI P.ch. α with the structure ethanolamine-phosphate-[Man(α 1-2)]Man(α 1-2)-Man(α 1-6)Man(α 1-4)anhydromannitol was eluted at 9 glucose units (results not shown). This shows that there is no major difference in size between the hydrophilic fragment of the free GPI P.ch. α and the corresponding fragment of the GPI peptide released from MSP-1. The Pronase-generated GPI peptide of MSP-1 probably has only one amino acid, as suggested by the minor difference in the elution behaviour between the hydrophilic fragment of P.ch. α and the respective fragment of the GPI peptide. The results suggest that *P. c. chabaudi* AS MSP-1 is either modified by the transfer of the GPI-anchor precursor P.ch. α or by the transfer of P.ch. β and the subsequent addition

Figure 5 Analysis of the MSP-1 GPI anchor

MSP-1 was immunoprecipitated from metabolically labelled blood-stage parasites. Non-covalently linked contaminants were removed by repeated organic solvent extraction before purification of the GPI peptide generated by Pronase treatment. The neutral core glycan generated by dephosphorylation, deamination and reduction from [^3H]glucosamine-labelled MSP-1 was analysed by HPAEC (Dionex) (**A**). The hydrophilic fragment generated by deamination and reduction was analysed by Bio-Gel P4 size-exclusion chromatography (**B**). Standards indicated correspond to the elution position of $\text{Man}(\alpha 1-2)\text{Man}(\alpha 1-2)\text{Man}(\alpha 1-6)\text{Man}(\alpha 1-4)\text{anhydromannitol}$ from rat brain Thy-1 GPI anchor (**A**) and partially hydrolysed dextran (**B**) respectively. The phosphatidylinositol fragment released from [^3H]myristic acid-labelled MSP-1 by HNO_2 deamination was analysed by TLC using solvent system A (**C**, lane A). The respective fragment of MSP-1 from *P. falciparum* was analysed in parallel (**C**, lane B). PI, migration position of phosphatidylinositol; acyl-PI, migration position of dimyristoyl-phosphatidyl(palmitoyl)inositol; O, origin; F, front.

of the fourth mannose found to modify the trimannosyl core glycan of the MSP-1 GPI anchor.

The hydrophobic fragment of the MSP-1 GPI anchor was investigated by analysing the phosphatidylinositol fragment released from [^3H]myristic acid-labelled MSP-1 by TLC using solvent system A (Figure 5C, lane A). The phosphatidylinositol fragment migrates slightly more hydrophobically than the dimyristoyl-phosphatidyl(palmitoyl)inositol standard generated from [^3H]myristic acid-labelled P3 (glycolipid C) of *T. b. brucei* and the corresponding fragment released from the MSP-1 GPI anchor of *P. falciparum* (Figure 5C, lane B). These data, in combination with the insensitivity of the MSP-1 GPI anchor to PI-PLC treatment and the sensitivity to alkaline treatment, imply that the protein-bound GPI anchor of MSP-1 carries an inositol ring that is modified by an ester-linked fatty acid and a diacylglycerol.

DISCUSSION

GPIs have been suggested to be a novel type of malarial toxin involved in cytokine release by host macrophages [1,3,4], the up-regulation of endothelial surface molecules [2] and insulin-mimetic activity in host cell adipocytes [1]. Most of these studies were carried out in cell culture systems, and the pathological effects of malarial GPIs *in vivo* have been identified to a lesser

extent [7]. One system that has been used to study malarial immunology and pathology is the rodent malarial parasite *P. c. chabaudi* (for example, see [26]). In this report we present the identification and characterization of free and protein-bound GPIs of this organism, to establish a basis for using it as a rodent model to study the pathology induced by malarial glycolipid toxins.

Using metabolic labelling techniques, we identified a set of glycolipids synthesized by *P. c. chabaudi* AS blood forms. TLC analysis of these glycolipids in comparison with GPIs already characterized in *P. falciparum* revealed almost identical patterns of [^3H]glucosamine-labelled glycolipids using two different TLC systems. The subsequent characterization of the individual TLC-purified *P. c. chabaudi* AS glycolipids by GPI-specific chemical and enzymic treatments showed that all but one of these glycolipids are GPIs with a modified inositol structure rendering them insensitive to PI-PLC treatment. Only P.ch. ζ is sensitive to PI-PLC treatment showing that the inositol present in this GPI is not modified.

For further structural analysis of these GPIs, neutral core glycans were generated by dephosphorylation, deamination and reduction of TLC-purified metabolically labelled glycolipids. This analysis revealed that *P. c. chabaudi* AS glycolipids represent a set of GPIs that are potential GPI-biosynthesis intermediates. We identified two non-mannosylated GPIs (P.ch. ζ and θ) with only glucosamine as the glycan part. As P.ch. ζ is sensitive to PI-PLC treatment whereas P.ch. θ is not and the two GPIs differ in their hydrophobicity based on TLC analysis, we propose that P.ch. ζ lacks the acyl modification at the inositol ring present in all other GPIs of *P. c. chabaudi* AS. Therefore we postulate the structure glucosamine-inositol-phosphate-diacylglycerol for P.ch. ζ and glucosamine-(acyl)inositol-phosphate-diacylglycerol for P.ch. θ . Based on the analysis of the neutral core glycans and chromatographic behaviour of the intact GPIs on TLC, we identified four differently mannosylated GPI-biosynthesis intermediates having one (P.ch. η), two (P.ch. ϵ), three (P.ch. δ) or four (P.ch. γ) α -mannose residues linked to the glucosamine-(acyl)inositol-phosphate-diacylglycerol found in all mannosylated GPIs of *P. c. chabaudi* AS. In addition, two hydrophilic GPIs were identified by TLC, which co-migrated with the GPI-anchor precursors P.f. α and β of the human malarial parasite *P. falciparum*. Structural characterization of the neutral core glycans of the two *P. c. chabaudi* AS GPIs using HPAEC and TLC analysis together with their sensitivity to treatment with specific α -mannosidases (Jack-bean and *A. saitoi*) showed that P.ch. α carries four and P.ch. β carries three α -mannose residues, with the terminal mannoses being in $\alpha 1,2$ -linkage. The ethanolamine phosphate characteristic for potential GPI-anchor precursors was shown to be attached to the third mannose of the trimannosyl core glycan. Analysis of the hydrophobic fragment of the potential GPI-anchor precursor P.ch. α showed that this GPI carries an acylated phosphatidylinositol moiety more hydrophobically than the dimyristoyl-phosphatidyl(palmitoyl)inositol found in P3 (glycolipid C) of *T. b. brucei*. These results, together with the alkaline sensitivity and the insensitivity to PI-PLC treatment, lead us to postulate the structure ethanolamine-phosphate-[($\alpha 1-2$)mannose]mannose($\alpha 1-2$)mannose($\alpha 1-6$)mannose($\alpha 1-4$)glucosamine-(acyl)inositol-phosphate-diacylglycerol for P.ch. α . On the basis of the data for the hydrophilic part of P.ch. β , the presence of a modification at the inositol ring and its chromatographic behaviour on TLC, we propose the structure ethanolamine-phosphate-mannose($\alpha 1-2$)mannose($\alpha 1-6$)mannose($\alpha 1-4$)glucosamine-(acyl)inositol-phosphate-diacylglycerol for P.ch. β .

Of the GPIs identified, P.ch. α and β are potential anchor

precursors to be transferred to protein. The structural characterization of the GPI anchor found on the MSP-1 of *P. c. chabaudi* AS suggests that P.ch. α serves as a GPI-anchor precursor or that P.ch. β is transferred to protein and subsequently modified by the transfer of a mannose. The GPI anchor of metabolically labelled MSP-1 was characterized as an acylglycerol-based GPI with an acylated inositol ring based on its sensitivity to HNO_2 deamination, alkaline saponification and GPI-PLD treatment and insensitivity PI-PLC treatment. The presence of an ester-linked fatty acid at the inositol ring and a diacylglycerol as membrane anchor was confirmed by analysis of the hydrophobic fragment released by HNO_2 deamination from [^3H]myristic acid-labelled MSP-1, showing that the protein-bound GPI also possesses a hydrophobic fragment that is more hydrophobic than the dimyristoyl-phosphatidyl(palmitoyl)inositol found in P3 (glycolipid C) of *T. b. brucei*. Detailed analysis of neutral core glycan using HPAEC revealed that the GPI anchor carries the evolutionarily conserved trimannosyl core glycan modified by an additional α -mannose. The elution position of the hydrophilic fragment on Bio-Gel P4 in comparison with the respective fragment of the GPI-anchor precursor P.ch. α showed that only one acid-labile ethanolamine phosphate is present in the protein-bound GPI anchor. The data presented in this paper show that *P. c. chabaudi* AS possesses a set of differently mannosylated GPI-biosynthesis intermediates and two potential GPI-anchor precursors. These findings are analogous with those reported for blood stages of the human malarial parasite *P. falciparum*. As *P. c. chabaudi* AS is frequently used as a rodent model of malaria and the GPI structures synthesized by these two parasites are identical, *P. c. chabaudi* AS should provide a good system for assessing the biological activities ascribed to GPIs of *P. falciparum* [1–4] and their potential role in severe human malaria (for a review see [27]). The structural characterization of *P. c. chabaudi* AS GPIs provides an essential basis for further experiments on the role of malarial GPIs in this rodent malaria model.

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