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Babesia bovis contains an abundant parasite-specific protein-free glycerophosphatidylinositol and the genes predicted for its assembly

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ABSTRACT

Autonomous glycosylphosphatidylinositol (GPI) molecules (also protein-free GPIs or free GPIs) have been reported to be particularly abundant in some parasitic protozoa and mediate strong immunomodulatory effects on the host immune system. In the work at hand we have investigated the existence of free GPIs in *Babesia bovis*. Comparative thin layer chromatographic analysis of the protein-free glycolipid fraction of *in vitro* cultured *B. bovis* merozoites and erythrocyte membranes demonstrated the presence of an abundant parasite-specific band. Its chemical analysis revealed a GPI species containing a chain of two mannose residues, N-glucosamine and non-acylated inositol. The lipid moiety linked to inositol was diacylglycerol. The total fatty acid composition showed predominantly long-carbon chain molecules (12% of $C_{22:0}$ and 45% of $C_{24:0}$). The potential of *B. bovis* to assemble the presented free GPI species was verified by the existence of seven genes in its genome that putatively encode the following GPI biosynthetic enzymes: PI N-acetyl-GlcN-transferase (PIG-A and GPI-1), N-acetyl-GlcN-PI-de-N-acetylase (PIG-L), acyltransferase (PIG-W), dolichyl-phosphate mannosyl transferase (DPM-1), GPI mannosyltransferase I (PIG-M), and GPI mannosyltransferase II (PIG-V). GPI biosynthesis is vital for the intraerythrocytic parasite stage as mannosamine, an inhibitor of GPI biosynthesis, impaired *in vitro* growth of *B. bovis* merozoites. Absence of the vast majority of N-glycan metabolism encoding genes in the *B. bovis* genome underscores that the growth inhibitory effect of mannosamine is attributable to its interference with GPI biosynthesis and not with assembly of N-linked oligosaccharides, as has been described for higher eukaryotes. Elucidation of the structure and biosynthesis of GPI may allow to facilitate the development of future immune interventions against bovine babesiosis.

1. Introduction

The hemoprotozoan pathogen *Babesia bovis* is the most virulent etiological cause of bovine babesiosis, a disease that hampers cattle production and causes great economic losses in tropical and subtropical areas worldwide (Bock et al., 2004). The identification of molecules critically involved in host–parasite relationships may lead to improved control strategies against protozoan infections. One such molecule is glycosylphosphatidylinositol (GPI), a glycolipid abundantly present in the membranes of pathogenic protozoa. In *Plasmodium*, *Trypanosoma*, *Leishmania* and *Toxoplasma*, GPI and its biosynthetic pathway have been intensively studied for the purpose of developing immune therapeutics and

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novel drugs (Smith et al., 1997; Ferguson, 1999, 2000; Kinoshita and Inoue, 2000; de Macedo et al., 2003). Autonomous and protein-anchoring GPI molecules described to date contain a unique constant basic structure. This structure is characterized by the presence of glucosamine, which is linked in position 1 to myo-inositol and in position 4 to the first of a chain of commonly three mannose residues. In GPI anchors, the third mannose is regularly found to be conjugated to the C terminus of a protein via phosphoethanolamine. Furthermore, a phospholipid is linked to the C-1 hydroxyl group of myo-inositol. This basic structure can be extended and ornamented in different ways. The phospholipid linked to the inositol moiety may consist of diacylglycerol, 1-alkyl-2-acylglycerol or ceramide. Other differences among GPI anchors include the presence or lack of inositol acylation, and the extent of additional phosphoethanolamine and sugar residues attached to the glycan core (McConville and Ferguson, 1993; Ferguson et al., 2008).

In *B. bovis*, GPI molecules were found to anchor a 42 kDa membrane-associated glycoprotein, by metabolic labeling with radioactive glucosamine and myristic acid (Hines et al., 1989). Later, it was observed that the predicted peptides of this and the other members of the Variable Merozoite Surface Antigen (VMSA) family, share a C-terminal GPI anchor attachment signal (Hines et al., 1992). This sequence feature has been found to be present in approximately 0.5–1% of genome-encoded proteins of lower and higher eukaryotes resulting in their prospective subsequent GPI modification (Eisenhaber et al., 2001, 2004; Orlean and Menon, 2007). Remarkably, it has been reported that in parasitic protozoa GPI-anchored proteins are hundred times more abundant on the cell surface than in higher eukaryotes (10^9 vs. 10^7), suggesting their structural and functional relevance (McConville and Ferguson, 1993; Ferguson et al., 1994; Ilgoutz et al., 1999; Nagamune et al., 2000; Ropert and Gazzinelli, 2000). Parasite GPI anchors appear to promote immune evasion by allowing the formation of a particularly dense outer monolayer of surface antigens (McConville and Ferguson, 1993). Moreover, in the case of *Trypanosoma brucei*, they allow the release of target surface proteins by action of phosphatidylinositol-specific phospholipase C (PI-PLC) (Blum et al., 1993).

Beyond protein-anchoring, a multitude of other functions related to free GPI species have been described in protozoan parasites. In these organisms, the existence of autonomous GPI in large quantities seems to be a hallmark, differentiating them from higher eukaryotes. In *Plasmodium* and *Leishmania*, free GPI molecules have been reported to be 4 and 10 times more abundant than protein-anchored GPIs, respectively, and they are a common major contributor to the parasite glycocalyx (McConville and Ferguson, 1993; Naik et al., 2000; Ropert and Gazzinelli, 2000).

Subject to its fine structure, GPI has been reported to exert strong immunomodulatory effects on the host immune system (Tachado et al., 1997, 1999). The large excess of GPI (malaria toxin) may lead, subsequent to *Plasmodium* infection, to GPI-dependent recognition by the host's immune system. Accordingly, it has been shown that GPI recognition by Toll-like receptors 2 and/or 4 mediate a strong proinflammatory cytokine response of host cells *in vitro* (Schofield and Hackett, 1993; Gowda, 2007). In line with these findings, *in vitro* and *in vivo* activation of both TLR2 and TLR4 by *Toxoplasma gondii* GPIs seems to be critical for eliciting a TNF-α response of innate immune cells in infections by this parasite (Debierre-Grockiego et al., 2007). In malaria, the observed potent induction of proinflammatory cytokines TNF-α, IFN-γ, IL-1, IL-6, and IL-12 is thought to be responsible for many of the severe pathogenic effects observed (Ropert and Gazzinelli, 2000; Schofield and Grau, 2005). Likewise, pattern and quantity of type-1 cytokine secretion following *B. bovis*-infection is the main suspected underlying cause for the severity of disease progression (Shoda et al., 2000; Goff et al., 2001; Goff et al., 2003). *B. bovis*-infection displays strikingly similar pathogenic symptoms as cerebral malaria and has therefore been proposed as an animal model for this disease (Krause et al., 2007).

As a basic prerequisite for future investigations on a possible role of *B. bovis* GPI in the pathogenicity of bovine babesiosis we report here on the analysis of: (i) the structure of a parasite-specific protein-free GPI species present in merozoites, (ii) the *in silico* identification of genes required for its biosynthesis, and (iii) the effect of specific inhibition of the GPI assembly pathway on the *in vitro* growth of these parasites.

2. Materials and methods

2.1. In vitro cultures of B. bovis merozoites

Merozoites of the *B. bovis* pathogenic strain S2P (Anziani et al., 1993) were *in vitro* grown, basically as described elsewhere (Ristic and Levy, 1980), in a medium containing 10% bovine erythrocytes and 40% normal bovine serum in M199 medium (Sigma–Aldrich, St. Louis, MO), supplemented with 5 mM HEPES (Sigma–Aldrich, St. Louis, MO), 100 μg/ml streptomycin, 100 UI/ml penicillin, pH 7.2. Cultures were grown in 24-well plates (in a depth of 0.6 mm), at 37 °C in a humidified 5% CO_2 atmosphere, with daily renovation of the overlaying medium. Percentages of infected erythrocytes (% IE) were estimated by microscopic examination of 3000 erythrocytes in Giemsa-stained smears. Subcultures were started when the percentage of infected erythrocytes (IE) was 5% or more.

2.2. Preparation of merozoite and erythrocyte membranes

A highly purified *B. bovis* merozoite suspension was obtained as follows. A 24 ml merozoite culture (60–90% IE) was left at 4 °C for 80 min, which yielded high amounts of free merozoite in the supernatant, and subjected to two low-speed centrifugation cycles (120 × *g*, 20 min, 4 °C), to pellet the erythrocytes, followed by centrifugation of the resulting supernatant at a higher speed (3000 × *g*, 30 min, 4 °C) to collect the merozoites. The merozoite pellet was suspended in PBS, vortexed and stored at −80 °C with 1 mM PMSF until use. An erythrocyte ghost suspension was used as control. An aliquot of 2.4 ml uninfected bovine erythrocytes was diluted 1/10 in a low ionic strength buffer (10 mM Tris–HCl, pH 7.0), incubated for 60 min on ice, and

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centrifuged at 23,400 × g, for 15 min at 4 °C. The pellet was suspended in PBS by vortexing and stored at −80 °C with 1 mM PMSF, until use.

2.3. Purification of the protein-free glycolipids

Basically, the protocol described by Naik et al. (2000) for *Plasmodium falciparum* GPI purification was employed. All procedures were carried out using acid-washed, siliconized glassware, as well as high quality solvents, and sterile water and buffers. Merozoite and erythrocyte membrane suspensions, obtained as described above, were lyophilized and extracted three times with 5 ml chloroform/methanol (2:1, v/v). This procedure caused the precipitation of the total glycosylated lipid fraction, which was then separated from non-glycosylated lipids by centrifugation. The extracts containing non-glycosylated lipids were evaporated, and their mass was determined by weight (21.3 and 20.3 mg for merozoite and erythrocyte lipids, respectively). The precipitated fraction was extracted with three consecutive portions of 3 ml of chloroform/methanol/water (10:10:3, v/v/v). These extracts were pooled and dried, and then partitioned between water and water-saturated butanol. The butanolic phase, containing protein-free glycolipids, was dried, dissolved in chloroform, and subjected to thin layer chromatography (TLC) on Silica Gel 60 F254 (Merck, Darmstadt, Germany), using chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v), as solvent mixture. Standards of phosphatidylinositol (PI) and bovine brain cerebroside sulfate (sulfatides, S), both from Sigma–Aldrich, were run on the same plate. Spots were visualized by exposure to iodine vapors. The TLC plate was scanned and the images analyzed with background correction using NIH ImageJ (http://rsb.info.nih.gov/ij).

2.4. Biochemical characterization of a B. bovis-specific protein-free GPI species

Spot 1 of the merozoite sample (Fig. 1) was scraped off the TLC plate, suspended in 0.1 ml of 50 mM Tris–HCl, pH 7.4, and digested with PI-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (Sigma–Aldrich, 0.35 U/reaction, 2 h, 37 °C), in the presence of 0.1% deoxycholate. Two-phase-extraction with chloroform/methanol (2:1, v/v) was used to separate the resulting lipid (organic phase) and glycan (aqueous phase) fractions. An aliquot of the lipid fraction was analyzed by TLC on Silica Gel 60 F254, using chloroform/methanol (19:1.5, v/v) as solvent mixture, and hexadecylglycerol (HG), ceramide (C), monoacylglycerol (MAG) and diacylglycerol (DAG), as standards. Detection was carried out by exposure to iodine vapors. Another aliquot was subjected to methanolysis by exposure to methanol/concentrated HCl/water (29:3:4, v/v/v) at 70–80 °C, for 18 h (Weiss et al., 1958). The acid was eliminated by successive evaporations with methanol, and fatty acids

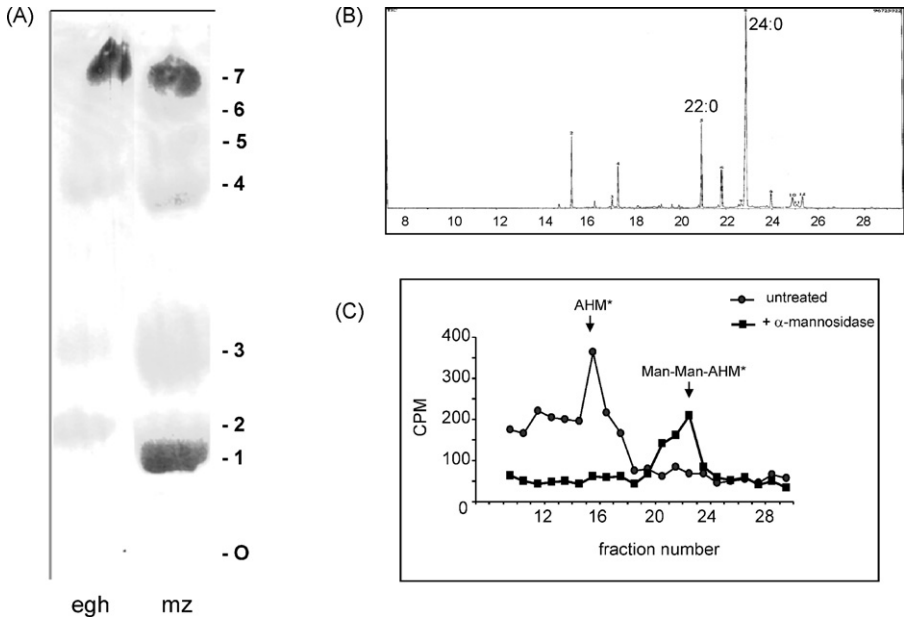

Fig. 1. Identification and structural analysis of a *B. bovis*-specific protein-free GPI. (A) Purification of protein-free GPI species by thin layer chromatography (TLC). Butanolic lipid extracts containing protein-free GPIs from *B. bovis* merozoites and bovine erythrocytes on Silica Gel 60 F254, using chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v). Lipid spots were visualized by exposure to iodine vapors. Spot 1 was further analyzed. Authentic standards of phosphatidylinositol and sulfosphingolipids were run on the same plate. Their Rf values coincide with spots 5 and 6, respectively. mz, merozoite membranes; egh, erythrocyte ghost membranes. (B) Fatty acid composition of Spot 1. Methyl ester derivatives of total Spot 1 fatty acids were analyzed by gas chromatography/mass spectrometry in a Shimadzu QP 5000-GC 17A instrument. The carbon lengths and number of unsaturations in each identified peak are shown. (C) Determination of mannose residues in Spot 1 glycan moiety. Spot 1 was treated with PI-PLC, and the glycans in the aqueous phase were analyzed by anion exchange chromatography in a HPAEC-PAD column, after conversion of GlcN into [^{3}H] AHM (AHM*). The plot shows the glycan elution profiles detected by scintillation counting, without (squares) and after (circles) treatment with α-1,4-mannosidase. Peaks were identified according to the elution profiles of glucose oligomer standards.

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were analyzed by gas chromatography/mass spectrometry (GC/MS) in a Shimadzu QP 5000-GC 17A instrument (Kyoto, Japan), equipped with a capillary column (Ultra-1, 25 m × 0.20 mm). The carrier gas was helium and the column temperature was initially set at 80 °C and raised to 290 °C, at a rate of 10 °C/min. Spectra were acquired from *m/z* 40–700. The glycan (aqueous) fraction, obtained after PI-PLC treatment and two-phase extraction of Spot 1, was freeze-dried and dissolved in 1 ml of 50 mM sodium acetate, pH 3.5. Solid sodium nitrite (2 mg) was added and after 3 h at room temperature, the mixture was neutralized with 1 M NaOH, and incubated with 100 μCi of [1-^{3}H] sodium borohydride (521 mCi/mmol; NEN, Boston, MA) for 2 h at room temperature. The labeling reaction was stopped by addition of solid sodium borohydride (1 mg), and after 2 h, the pH was lowered to 5 with 0.1 M acetic acid. The sample was desalted using Dowex 50 (H^+) (The Dow Chemical Co., Midland, MI), and lyophilized. After reconstituting the lyophilate with 60 μl water (sample A), an aliquot (20 μl) was subjected to high pH anion exchange chromatography on a Dionex BioLC system (Dionex Corporation, Sunnyvale, USA) fitted with a Carbo-Pack PA10 anion exchange column, a PA-10 precolumn, and a pulsed amperometric detector (PAD). AHM species bound to 0–4 mannose (Man) molecules were eluted at a flow rate of 0.6 ml/min, using a pH gradient formed as follows: (i) 95% solution A (0.15 M NaOH); (ii) 80% solution A/20% solution B (0.25 M sodium acetate in 0.15 M NaOH) for 50 min; and (iii) a wash cycle with 100% solution B at 55 min held for 15 min (Agusti et al., 1998). Eluate fractions were collected every 30 s and neutralized with 2 M sodium acetate. The radioactivity in each fraction was measured in a scintillation counter after addition of 1 ml of scintillation fluid (4 g/l PPO and 40 mg/l POPOP in Arcopal X-100/Toluen, 1:3 v/v). The elution time of AHM, ManAHM, Man_2AHM, Man_3AHM and Man_4AHM was calculated using glucose oligomer standards, as described by Güther et al. (1992). Another 20 μl aliquot of sample A was lyophilized and treated with 0.1 U of jack bean α-mannosidase (Sigma–Aldrich) in 0.1 M sodium acetate, pH 5, for 2 h, at room temperature, followed by 22 h at 37 °C. The mixture was boiled for 1 min for enzyme inactivation, desalted and analyzed as described above by anion exchange chromatography and liquid scintillation counting.

2.5. In silico identification of B. bovis genes putatively involved in GPI and N-glycan synthesis

A BLASTp (http://blast.ncbi.nlm.nih.gov) search of the *B. bovis*, *Theileria annulata* and *Theileria parva* genomes was carried out using the amino acid sequences of predicted or functionally characterized *P. falciparum* GPI biosynthetic enzymes (Gardner et al., 2005; Pain et al., 2005; Brayton et al., 2007). Orthology of *B. bovis* with *P. falciparum*, *T. parva and T. annulata* amino acid sequences, as well as with well characterized homologous sequences from *Homo sapiens*, and *Saccharomyces* sp. identified in the GenBank, was confirmed by a reverse BLAST test. For each enzyme a global alignment of all amino acid sequences was performed with a BLOSUM50 matrix (gap penalty 12, gap extension penalty 4), and the identity between sequence pairs calculated (MatGAT program, Campanella et al., 2004). A BLASTp search of the *B. bovis* reference protein and a tBLASTn search of the *B. bovis* reference genome database with amino acid sequences of the 13 well characterized Alg (asparagine-linked glycan) glycosyltransferases of *Saccharomyces cerevisiae* Alg1 to Alg3, and Alg5 to Alg14, as well as with the presumed flippase associated protein Rft1, and oligosaccharidetransferase STT3 was performed.

2.6. B. bovis in vitro growth experiments in the presence of mannosamine

Aliquots (100 μl) of a 72 h merozoite culture with 8% IE were distributed in several wells of a 96-well microplate, containing 0.1 ml culture medium + different concentrations (0, 0.1, 0.3, 3, and 10 mM) of ManN (Sigma–Aldrich). ManN was added from a 1 M stock solution in 199 medium. Each ManN concentration was tested in triplicate wells. Uninfected bovine erythrocytes were added at time 0 to a final concentration of 10% (v/v). The overlay was daily changed with fresh medium containing ManN, so that the inhibitor concentration remained constant during the experiment. After 72 h, the % IE in each well was determined by microscopic counting of 3000 erythrocytes in Giemsa-stained smears. The statistical significance of the differences between the mean % IE obtained at each ManN concentration with respect to the untreated control was calculated using the Student's *t*-test.

3. Results and discussion

3.1. Biochemical analysis of a B. bovis-specific protein-free GPI

Thin layer chromatographic separation of lipid extracts containing the protein-free glycolipids of merozoite and bovine erythrocyte membranes, allowed the identification of a *B. bovis*-specific spot absent in the erythrocyte membrane sample (Spot 1, Fig. 1A). Spot 1 was sensitive to PI-PLC treatment, yielding diacylglycerol (DAG) as the only lipidic product (data not shown). Since hydrolysis by PI-PLC is impeded by the existence of acyl moieties at position 1 of inositol, it is to be concluded that the inositol moiety of Spot 1 PI is non-acylated (Roberts et al., 1988). Fatty acids isolated from Spot 1 were analyzed by GC–MS. Most remarkably, long-chain fatty acids behenic acid ($C_{22:0}$) and lignoceric acid ($C_{24:0}$) were found in predominant quantities of 12 and 45%, respectively (Fig. 1B). To analyze the glycan moiety of the PI-PLC treated Spot 1, GlcN was converted into ^{3}H-labeled anhidromanitol (AHM*), and the number of mannose residues attached analyzed by high pH anion exchange chromatography (Fig. 1C). The main peak was identified as di-mannose-AHM (Man_2AHM), corresponding to a glycan structure of Man_2-GlcN. These results were verified by hydrolysis with jack bean α-mannosidase yielding, as expected, a single peak of AHM* (Fig. 1C). Taken together, the above investigation demonstrates that Spot 1 contains a *B. bovis*-specific protein-free GPI species with a structure of: Man-Man-GlcN-inositol-diacylglycerol that contains predominantly long-chain fatty acids $C_{22:0}$ and $C_{24:0}$.

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Several other spots were observed in the TLC plate of Fig. 1A. Spots 3 and 4 were each more than two times increased in the merozoite as compared to the erythrocyte sample, while Spot 2 was present in similar amounts in both. Preliminary investigation of merozoite Spots 3 and 4 by PI-PLC treatment followed by analysis of the glycan moieties by labeling, treatment with mannosidase and liquid chromatography yielded a peak of AHM*, indicating that these spots also contain protein-free GPIs. However, since it was not clear whether these compounds are *B. bovis*-specific, they were not further investigated in this work. Spots 5 and 6 showed the same Rf values as phosphatidylinositol and sulfoglycosphingolipids standards, respectively. The latter have been demonstrated for *P. falciparum* (Landoni et al., 2007), but as yet their identification in *B. bovis* membranes has not been reported. Spot 7 is presumed to contain neutral lipids.

Compared to other eukaryotic systems, and in particular, to the closely related *P. falciparum*, the *B. bovis* free GPI species is unusual with respect to three structural features. First, its high abundance suggests that it represents a final metabolic product, while in other organisms, free GPI species with two mannoses are usually transient metabolic intermediates that are present in minor quantities. As to our knowledge, in exclusively one case, a free GPI with a resembling structure (Man-Man-GlcN-inositol-alkylacylglycerol) has been described as the major component of the cell surface of the amastigote stage of *Leishmania donovani* (type-1 GIPL: M2) (McConville and Blackwell, 1991). In *P. falciparum*, on the other hand, the major component of the free GPI fraction contains four mannoses, while a three-mannose variety is also present in lesser amount (Schmidt et al., 1998; Naik et al., 2003). The second structural peculiarity of the *B. bovis* free GPI is that its inositol is not acylated. In contrast, in *P. falciparum*, all free and protein-linked GPI structures so far characterized were found to contain acylated inositol (Gerold et al., 1996, 1999; Naik et al., 2000). Finally, the predominance of long-chain fatty acids $C_{22:0}$ and $C_{24:0}$ in the DAG lipid moiety is highly unusual. Although the lipidic core of *P. falciparum* consists also of DAG, very low levels of $C_{22:0}$, and absence of $C_{24:0}$ were reported. For accumulation of these long-chain fatty acids, *B. bovis* either needs to have special capabilities for their synthesis, or the ability to scavenge them from the host.

3.2. *B. bovis* genes predicted to participate in GPI biosynthesis

To analyze whether *B. bovis* contains the biosynthetic machinery to synthesize the characterized free GPI species (Man-Man-GlcN-inositol-diacylglycerol), data mining of the *B. bovis* genome was carried out to find the required GPI biosynthetic enzymes. The enzymatic steps leading to a two-mannose GPI structure, based on the postulated pathway for *P. falciparum* are depicted in Fig. 2 (Gerold et al., 1994; Delorenzi et al., 2002). With the exception of the flippase of step 3, which has not been identified in any organism so far, genes encoding all the postulated GPI biosynthetic enzymes could be found in the *B. bovis* genome, using predicted or experimentally verified *P. falciparum* enzyme sequences (Delorenzi et al., 2002; Shams-Eldin et al., 2002). The respective pathway steps, enzymatic activities, and the accession numbers of the encoding *B. bovis* genes are shown in Table 1.

In addition, these genes could also be found in the genomes of the related piroplasmids *T. parva* and *T. annulata* (Table 1). Global alignments of *B. bovis* with *T. annulata*, *T. parva*, *P. falciparum*, *S. cerevisiae*, *H. sapiens* orthologs of each group of protein sequences were inspected for the presence of signature motifs as characterized and reported by Delorenzi et al. (2002). The respective signatures were found to be highly conserved in all analyzed organisms and could be unequivocally verified

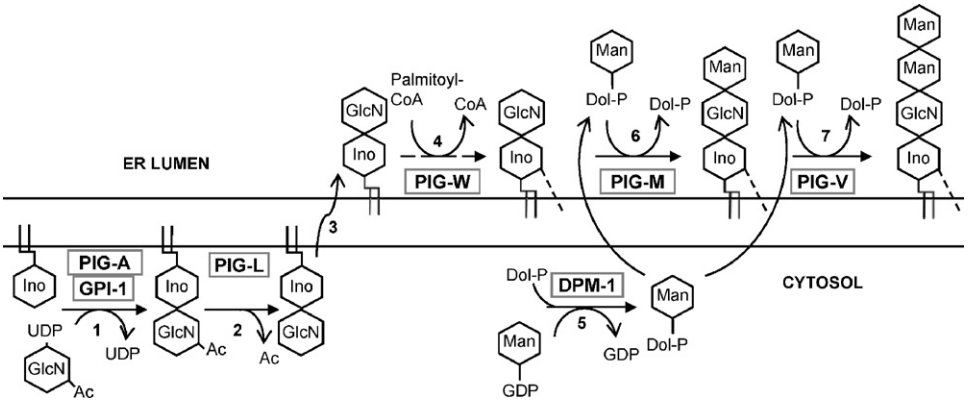

Fig. 2. Assembly of a two-mannose GPI molecule according to the postulated GPI biosynthetic pathway of *P. falciparum*. Steps 1 and 2 of GPI biosynthesis take place in the cytoplasmic side of the ER. First, UDP-N-acetylglucosamine (GlcNac) and PI are linked to form GlcNAc-PI, catalyzed by a complex formed by PIG-A and GPI1 (step 1). Removal of the N-acetyl group is catalyzed by PIG-L and yields GlcN-PI (step 2). GlcN-PI is translocated to the luminal ER side by a so far uncharacterized flippase (step 3). The following step (4), catalyzed by PIG-W, involves the addition of a fatty acid to the inositol ring of PI, resulting in GlcN-acyl-PI. However, this step appears to be non-operative in the synthesis of the identified *B. bovis* free GPI species. Two mannoses are then sequentially added to GlcN-acyl-PI (or GlcN-PI), catalyzed by PIG-M and PIG-V (steps 6 and 7). The mannose donor substrate is dolichol-phosphate-mannose, which is synthesized in the cytoplasmic side by DPM1 from dolichol-phosphate and GDP-mannose (step 5) and then transported to the luminal side across the membrane.

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Table 1
Identification of *B. bovis* genes predicted to participate in the GPI biosynthetic pathway. Enzymatic activities and genes as identified in *P. falciparum*, orthologous *B. bovis* genes and gene product lengths, as well as percent identities between *B. bovis* enzymes and orthologs of *Plasmodium falciparum* (*Pf*), *Theileria parva* (*Tp*), *Theileria annulata* (*Ta*), *Homo sapiens* (*Hs*) and *Saccharomyces cerevisiae* (*Sc*) are given.

Step	Enzyme	Gene	*B. bovis* gene (acc. no.)	*B. bovis* product length (aa)	Identity (%)				
					vs. *Pf*	vs. *Tp*	vs. *Ta*	vs. *Hs*	vs. *Sc*
1	Glycosyltransferase	PIG-A	BBOV-III008560	397	33.3	40	39	33.7	36.2
		GPI-1	BBOV-III009660	256	12.1	14.9	14	12.6	11.8
2	de-N-acetylase	PIG-L	BBOV-III007080	253	31.7	28.8	25.1	29	24
3	Flippase[a]	nd	nd	nd	nd	nd	nd	nd	nd
4	Acyltransferase	PIG-W	BBOV_I001780	200	14.0	18	16.9	13.9	13.2
5	Dol-P-Man synthase	DPM-1	BBOV-III011210	253	43.2	36.0	41.3	43.8	29.4
6	Mannosyltransferase MT-I	PIG-M	BBOV-II000550	402	41.8	41.4	40.9	34.4	34.3
7	Mannosyltransferase MT-II	PIG-V	BBOV-IV007380	537	22.6	18.8	22.0	20.9	19.8

nd, not determined.
[a] The existence of this enzyme has been implied.

in the *B. bovis* amino acid sequences corresponding to PIG-A, GPI-1, PIG-L, DPM-1, and PIG-M genes (Table 1, data not shown).

In *P. falciparum*, only GPI-1 among the above-mentioned enzymes was functionally characterized by complementation of a yeast mutant deficient in GPI synthesis (Shams-Eldin et al., 2002). More recently, PIG-V and PIG-W, that had not been reported by Delorenzi et al. (2002), were identified based on functional analysis in yeast (Murakami et al., 2003; Kang et al., 2005). The catalytically active amino acid residues of the mannosyltransferase II encoded by PIG-V are well characterized and, after alignment, they were also found to be conserved in the *B. bovis* PIG-V sequence (data not shown).

GWT1, the yeast ortholog of PIG-W, catalyzes the acylation of GlcN-inositol-PI to generate GlcN-(acyl)inositol-PI and was shown to be critical for GPI-anchored protein expression (Murakami et al., 2003; Umemura et al., 2003). This seems to be the case also in *Plasmodium* sp., where inositol acylation is a prerequisite for subsequent mannosylation of GPI and treatment with GlcN, an inhibitor of inositol acylation, resulted in growth arrest at the trophozoite stage (Gerold et al., 1999; Naik et al., 2003). By BLASTp search with yeast GWT-1 (*Sc*PIG-W), we were able to identify the PIG-W ortholog of *B. bovis* (*Bb*PIG-W). However, compared to yeast GWT-1 and other PIG-W members, which commonly exhibit sequence lengths of about 500 aa, *Bb*PIG-W covers only the C-terminal 201 aa, raising doubts with regard to its functionality. Interestingly, the *T. parva* (302 aa) and *T. annulata* (390 aa) PIG-W sequences are also substantially shorter than other members of this protein family, suggesting that this is a common characteristic of piroplasmids. Acylation step 4 of the postulated biosynthetic pathway is, apparently, not involved in the assembly of the characterized *B. bovis* free GPI compound as inositol was found to be non-acylated (Fig. 2). This might suggest that *Bb*PIG-W is non-functional or that it is engaged at a later step of the pathway. The latter possibility seems to be more likely in view of a report in which inositol palmitoylation of a GPI-anchor in *Babesia divergens* was suggested (Delbecq et al., 2002).

As outlined in Section 3.1, the characterized *B. bovis* GPI species (Man_2-GlcN-inositol-DAG) is parasite specific and probably represents the major free GPI component. Consistent with this view is that PIG-B, which catalyzes the addition of the third mannose residues in GPI, could not be found in the *B. bovis* genome suggesting that the identified GPI species may be a final metabolic product (Basagoudanavar et al., 2007). This notion is further supported by our failure to identify PIG-B in the genomes of *T. annulata* and *T. parva* as well, suggesting that absence of PIG-B and, hence, lack of a third mannose in GPIs of these parasites may be a common trait of piroplasmid protozoans.

3.3. Mannosamine inhibition of *B. bovis* merozoite growth

B. bovis merozoites were *in vitro* cultured in the presence of different concentrations of mannosamine (ManN) reported to act as an inhibitor of GPI biosynthesis in higher eukaryotic cells and in protozoan parasites such as *Leishmania mexicana*, *T. brucei*, and *P. falciparum* (Lisanti et al., 1991; Pan et al., 1992a; Field et al., 1993; Ralton et al., 1993; Naik et al., 2000). Accordingly, an inhibitory effect on *B. bovis* growth was also observed in ManN-treated as compared to untreated cultures. Fig. 3 shows the percentage of infected erythrocytes (% IE) at the initiation

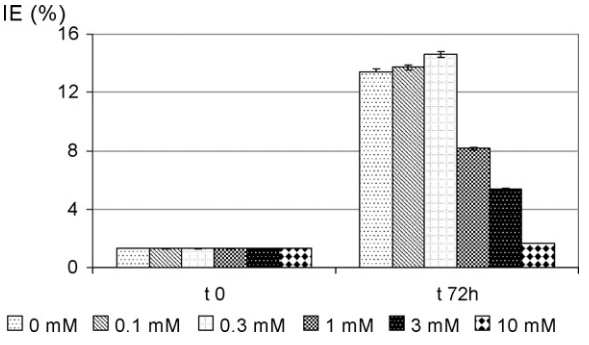

Fig. 3. Mannosamine (ManN) inhibits the *in vitro* growth of *Babesia bovis* merozoites. Merozoites of *B. bovis*, strain S2P, were *in vitro* grown for 3 days in the presence of different concentrations of ManN (0, 0.1, 0.3, 3, and 10 mM). The percentages of infected erythrocytes (% IE) were measured at the beginning of the experiment (*t*0) and after 72 h as determined by microscopic observation of Giemsa-stained blood smears. The graph shows the average number of infected erythrocytes in percent at each ManN concentration of three parallel cultures ±SD.

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of cultures at day 0 and after 3 days of growth, as assessed by microscopic observation of stained smears. Control cultures grew from an average initial parasitemia of about 1% up to 13%. On the other hand, *B. bovis* cultures treated with 1, 3 and 10 mM ManN showed a dose-dependent significant ($p < 0.001$) growth inhibition of 41, 66 and 97%, respectively. These ManN concentrations were similar to those reported to be effective for *P. falciparum* (Naik et al., 2000; de Macedo et al., 2001; Naik et al., 2003). In spite of the observed differences in *B. bovis* growth, around 85% of the parasites inside erythrocytes displayed the typical pear shape and showed the expected distribution of two merozoites per erythrocyte, both in control and treated samples, underscoring that ManN is not toxic for this parasite.

The mechanism of inhibition of GPI biosynthesis by ManN was first reported in Madin–Darby-canine kidney cells and *L. mexicana* where the inhibitor was found to be incorporated to form ManN-Man-GlcN-PI, an aberrant substrate that accumulates as it cannot be further metabolized (Lisanti et al., 1991; Pan et al., 1992a; Field et al., 1993; Ralton et al., 1993). Later investigations showed that ManN also inhibits GPI mannosyltransferase 3 (MT-III) in *T. brucei*, prevents incorporation of the first ManN into GlcN-PI in *P. falciparum*, and can be converted into the epimer GlcN in both these parasites (Ralton et al., 1993; Naik et al., 2000; de Macedo et al., 2001). However, GlcN inhibits GPI assembly in a similar dose-dependent mode as ManN, since it specifically impedes the formation of GlcN-(acyl)PI through inhibition of inositol acylation in *Plasmodium* (Naik et al., 2003). Thus, although ManN seems to target GPI biosynthesis at different steps and levels, it has been shown to prevent exclusively GPI assembly without affecting total protein biosynthesis in all eukaryotic systems investigated so far (de Macedo et al., 2001). Furthermore, inhibitory effects observed on *P. falciparum* were not due to nonspecific ManN toxicity as withdrawal restored common growth patterns, and only the trophozoite stage, during which GPI anchor biosynthesis takes place, was affected (Naik et al., 2000). This is in agreement with the results presented in this report, where ManN arrested *B. bovis* growth but did not seem to be toxic, since the parasite shape remained unchanged.

In higher eukaryotes, ManN has been described to affect other pathways as well, such as the assembly of N-linked oligosaccharides (Pan and Elbein, 1985; Pan et al., 1992b). It could therefore be suspected that the observed effect of ManN in *B. bovis* is caused by inhibition of N-glycan formation. To understand the degree of N-glycosylation in this parasite, the existence of genes encoding Alg glycosyltransferases (Alg, Asn-linked glycan), the possible accessory flippase protein (Rft1), and oligosaccharyltransferase (STT3) in the *B. bovis* genome were explored (Helenius et al., 2002; Frank et al., 2008; Stanley et al., 2008). Of these, exclusively Alg7 (acc. no. XP_001609261), Alg 13/14 (acc. no. XP_001610083 and XP_001610338, respectively), and STT3 (acc. no. XP_001609551) could be identified. Most noteworthy, no Alg mannosyltransferases as well as no other enzymes that participate in the pathway were found after a thorough search of the *B. bovis* genome, and do most likely not exist in this organism (data not shown). In view of these findings it seems that *B. bovis* possesses, if any, only highly restricted capabilities of N-glycosylation. Common ancestry has been reported to be highly predictive for the inventory of Alg glycosyltransferases and accordingly, other protozoans such as *Plasmodium* and *Giardia* spp., contain the same restricted Alg enzyme repertoire (Samuelson et al., 2005; Mendonca-Previato et al., 2005; von Itzstein et al., 2008). With regard to Plasmodium, this correlates well with the report of the existence of N-glycosylation of proteins by Kimura et al. (1996), and the later report by Gowda et al. (1997) that this process occurs to a very limited extent. Based on these observations, the effect of ManN on *B. bovis* growth is most likely due to interference with GPI assembly than with N-glycosylation. Thus, these studies indicate that GPI biosynthesis is vital for the intraerythrocytic merozoite stage of this parasite.

The work at hand allows a first insight into the glycobiology of *B. bovis*, a research area that will improve our understanding of the pathobiology of related piroplasmids and other tick-transmitted hemoparasites, and thereby have an impact on vaccine development and other therapeutic approaches.

Conflict of interest statement

The authors declare no conflicts of interest.

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