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Original article

The glycosylphosphatidylinositol-anchored protein repertoire of *Babesia bovis* and its significance for erythrocyte invasion

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ABSTRACT

Glycosylphosphatidylinositol-anchored proteins are abundant on the surface of pathogenic protozoans and might play an important role for parasite survival. In the present work, the relevance of GPI-anchored proteins for erythrocyte invasion of the cattle hemoparasite *Babesia bovis* was studied. We show that cleavage of GPI-anchored antigens from the merozoite parasite stage by phosphatidylinositol-specific phospholipase C abolished invasion of erythrocytes demonstrating the importance of this class of molecules for parasite propagation. In addition, the repertoire of GPI-anchored proteins of *B. bovis* was predicted with high fidelity by searching its genome with available web-based bioinformatic tools. Altogether 17 GPI-anchored proteins were identified, 5 of which represent the already characterized variable merozoite surface antigens (VMSAs). Fifteen of the identified GPI-anchored proteins contain 2–26 amino acid repeats indicating that they are likely involved in functions of recognition, adhesion, or transport. Repeats were found to contain an increased frequency of proline, indicative of unstructured regions; and were estimated to be 3.21 times more hydrophilic than non-repeat regions. This suggests that they might represent eminent antibody epitopes. The majority of the putative GPI-anchored antigens reported in this work have so far remained unnoticed, though they may represent potential candidates for inclusion in a subunit vaccine.

Introduction

The tick-transmitted hemoparasite *Babesia bovis* is a major causative agent of bovine babesiosis, an often fatal disease of cattle that is endemic in tropical and subtropical regions worldwide (Bock et al., 2004). Currently, in many countries control of bovine babesiosis is primarily secured by vaccination using attenuated parasites. In order to overcome the intrinsic drawbacks of existent live vaccines, efforts are made to identify parasite molecules for the development of non-live vaccines (Brown et al., 2006).

Prominent vaccine candidates of many pathogenic apicomplexan protozoans are surface proteins containing glycosylphosphatidylinositol (GPI) anchors. Protection against *Toxoplasma gondii* infection was achieved by vaccinating mice with adenovirus constructs that encode the GPI-anchored primary surface antigens (SAGs) of this parasite (Caetano et al., 2006). In *Plasmodium falciparum*, the principal GPI-anchored surface proteins of sporozoites and schizonts, the circumsporozoite protein (CSP) and the major surface proteins (MSP-1 and -2), respectively, have been shown to confer variable degrees or even complete protection against challenge in rodent models (Holder and Freeman, 1981; Plassmeyer et al., 2009; Reece et al., 2004). Additionally, it has been shown that recombinant Bd37, a GPI-anchored protein of *Babesia divergens*, protected gerbils against a heterologous challenge with this parasite (Hadj-Kaddour et al., 2007). In *B. bovis*, the variable merozoite surface antigens (VMSAs) have been proposed to represent important candidates for inclusion in possible future recombinant vaccines. They are expressed on the merozoite and sporozoite surface, and antibodies against them have been shown to neutralize in vitro erythrocyte invasion (Carcy et al., 2006;

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Florin-Christensen et al., 2002; Hines et al., 1992, 1995; Mosqueda et al., 2002a; Suarez et al., 2000; Wilkowsky et al., 2003).

In contrast to surface proteins that are anchored into the membrane with a transmembrane domain, GPI-anchored proteins are attached to the cellular membrane via their lipid component. Apart from the lipid moiety, the core GPI structure is composed of myoinositol, N-acetylglucosamine, and generally 3 mannose groups that are linked via phosphoethanolamine to the C-terminal end of a protein (Ferguson et al., 2009). Importantly, the GPI-anchor can be enzymatically removed with phosphatidylinositol-specific phospholipases C (PI-PLC) converting the protein in its water soluble form and releasing it into the extracellular medium. Proteins destined to be GPI-anchored share as features (i) an N-terminal signal sequence targeting them to the endoplasmic reticulum (ER), (ii) a C-terminal transmembrane domain (TM) for transient anchoring in the ER membrane, and (iii) an ω cleavage site at which the protein is linked to the GPI moiety. The ω site is situated a few amino acids before the C-terminal hydrophobic domain which, upon cleavage, is replaced by the GPI anchor (Udenfriend and Kodukula, 1995).

GPI-anchored proteins are present in all eukaryotic cells, but it has been reported that they can be a hundred times more prevalent on the cell surface of pathogenic protozoans suggesting a salient role in these organisms (McConville and Ferguson, 1993). It has been put forward that the smaller dimension of the GPI anchor may allow for denser packing of surface antigens without compromising the integrity of the cellular membrane thus protecting the parasite from the immune attack of the host by the formation of protective coats (Ferguson, 1999). In addition, GPI-anchored proteins are thought to display an increased lateral mobility as compared to transmembrane proteins, a feature that may be important for cellular motility and host cell invasion (Ferguson et al., 2009; Low, 1989). Evidently, GPI-anchored proteins are highly accessible surface molecules and are therefore intimately involved in a variety of host–parasite interactions. In pathogenic protozoans, GPI-anchored proteins have been implicated in the formation of protective coats, host cell adhesion, recognition, and invasion (Ferguson et al., 2009). Other likely functions are connected to transport and signaling (Robinson, 1997; Robinson et al., 1989). The involvement in these pivotal mechanisms of parasite survival makes them primary candidates for vaccine development.

The objective of the work in hand is (i) to demonstrate the involvement of the GPI-anchored proteome expressed on the merozoite surface for erythrocyte invasion, a crucial step in the life cycle of *B. bovis*, (ii) to in silico identify the GPI-anchored protein repertoire encoded by *B. bovis* combining the predictive power of several bioinformatic web-based systems, (iii) to gain an improved insight of the potential function of identified GPI-anchored proteins by determining the existence of amino acid repeats and amino acid biases between repeat and non-repeat regions, and (iv) to investigate their genome location and paralogous relatives.

Materials and methods

Parasite cultures

Babesia bovis merozoites of the Argentine S2P pathogenic strain were in vitro cultured on bovine erythrocytes, in 75-cm^2 tissue-culture bottles, basically as described by Levy and Ristic (1980). Erythrocytes were aseptically isolated from defibrinated bovine blood after 3 washes using VYM buffer (0.016 g/l $CaCl_2 \cdot 2H_2O$; 0.4 g/l KCl; 1.45 g/l KH_2PO_4; 0.77 g/l Na_2HPO_4; 0.1 g/l $MgSO_4 \cdot 7H_2O$; 7 g/l NaCl; 20.5 g/l glucose; 0.0423 g/l adenine; 0.0708 g/l guanosine; pH 7). Erythrocytes suspended 1:2 in VYM were kept at 4 °C and used within 30 days. The basic culture medium (BCM) consisted in M199 (Sigma–Aldrich, St. Louis, MO), 5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid] (Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, pH 7.2. This medium was supplemented with normal bovine serum from the same donor of erythrocytes in a proportion of BCM to serum of 60:40 (v/v) (BCM-S). Cultures were grown on 10% (v/v) bovine erythrocytes and 90% BCM-S at 37 °C in a 5% CO_2 atmosphere, with daily changes of BCM-S, using a starting percentage of 1–2% infect erythrocytes (IE). Growth was monitored by microscopic observation of Giemsa-stained smears. For maintenance, cultures were split with addition of fresh erythrocytes every 48–72 h. In order to get an erythrocyte suspension with a higher parasitemia, a culture was diluted 1:2 on 2 consecutive days by addition of an equal volume of BCM-S and incubated as above. The resulting suspension had no less than 15% IE and was used as starting material for the purification of *B. bovis* merozoites.

Purification of B. bovis merozoites

A suspension (240 ml) of erythrocytes showing 15% infection with *B. bovis* merozoites, obtained as described above, was exposed to 4 °C for 2 h. At this temperature, merozoites accumulate outside erythrocytes and can be highly enriched by differential centrifugation (Rodriguez et al., 1986). The erythrocytes were pelleted by low-speed centrifugation (300 × *g*, 10 min, 4 °C), and the supernatant containing extracellular merozoites was separated and centrifuged (3000 × *g*, 20 min, 4 °C). Merozoites were washed twice by centrifugation in BCM, suspended in 0.2 ml of BCM-S, and quantified in a Neubauer hematocytometer. This concentrated suspension of free merozoites contained a low number of infected and non-infected erythrocytes. To avoid jeopardizing the viability of the highly delicate merozoites, additional low-speed centrifugation steps that might have further reduced the level of erythrocytes were dismissed.

Treatment of B. bovis merozoites with phospholipase C

The extracellular merozoites suspension obtained as described above was divided in equal aliquots (each containing 7.47×10^6 merozoites), one of which received 1 U/ml of *Bacillus thuringiensis* PI-specific phospholipase C (Sigma–Aldrich). The amount of PI-PLC was similar to that used by Coonrod et al. (1999) in live mouse oocytes. Both aliquots were incubated for 80 min at 37 °C, after which they were centrifuged and suspended in 1.5 ml of BCM-S. In order to determine merozoite viability, an additional smaller aliquot (50 μl) was taken from the PI-PLC-treated and also from the untreated merozoite suspension. Both aliquots were washed by centrifugation and incubated in 0.4 mg/ml propidium iodide in 1× PBS for 20 min at room temperature in the dark. The 2 aliquots were washed 4 times (3000 × *g*, 10 min, 4 °C), and resuspended in 1× PBS. Viability was estimated by counting the total number of merozoites by light microscopy vs. the number of dead (propidium iodide-stained) merozoites by fluorescence microscopy using a 1000× magnification. PI-PLC-treated and non-treated merozoite suspensions were divided in 0.15-ml aliquots in a 96-well microtiter plate, each of which contained 35×10^4 motile merozoites. Uninfected bovine erythrocytes washed in VYM buffer were quantified in a Neubauer chamber and added to the wells at a 1:1 merozoites/erythrocytes ratio. Plates were incubated at 37 °C, in a 5% CO_2 humidified atmosphere. At 0, 4, and 18 h, triplicate wells for each condition were separately centrifuged, and the whole pellets were smeared in microscopic slides and stained with Giemsa. IE percentages were calculated by microscopic examination of 3500 erythrocytes per smear using 1000× magnification.

Bioinformatic tools used for predictions and analysis

The following bioinformatic tools were used to identify GPI anchored-proteins in the *B. bovis*, strain T2Bo, predicted proteome (Brayton et al., 2007): GPI-SOM (Fankhauser and Maser, 2005), big-PI (Eisenhaber et al., 2004), Pred-GPI (Pierleoni et al., 2008), and FragAnchor (Poisson et al., 2007). In addition, SignalP 4.1 was used to identify signal peptides based on Neural Network and Hidden Markov model algorithms (Bendtsen et al., 2004; Petersen et al., 2011). TOPCONS and DAS were used to identify a C-terminal transmembrane domain (Bernsel et al., 2009; Cserzo et al., 1997). Amino acid repeats in GPI-anchored proteins were detected using the rapid automatic detection and alignment of repeats tool (RADAR) (Heger and Holm, 2000). Using ProtParam, the percentage of amino acids in repetitive and non-repetitive sequences and the GRAVY value was determined. The latter is an estimate of the sum of the hydropathicity value for each amino acid residue divided by the length of the sequence (Gasteiger et al., 2005; Kyte and Doolittle, 1982).

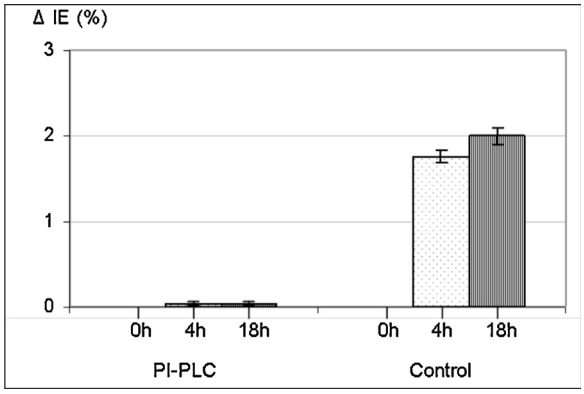

Fig. 1. Inhibition of merozoite invasion of bovine erythrocytes by GPI anchor cleavage. PI-PLC-treated (PI-PLC) or non-treated (control) live merozoites were incubated with equal numbers of bovine erythrocytes. The increase of infected erythrocytes (ΔIE) after 0 h, 4 h, and 18 h is shown. Results show the average of triplicate parallel suspensions ±SE. The results are representative of 2 independent experiments.

Results and discussion

Cleavage of merozoite GPI-anchored proteins leads to inhibition of erythrocyte invasion

It has been previously experimentally demonstrated that the MSA-1 of *B. bovis* contains a functional GPI anchor located at the C-terminal sequence of the molecule. Importantly, MSA-1 shares an identical GPI anchor signal region located at the C-terminal sequence with other members of the VMSA family, including MSA-2c (Hines et al., 1989). The presence of a fully conserved GPI signal in all members of the VMSA family strongly suggests that all VMSA members are anchored via GPI molecules in the surface membrane. To demonstrate the functional relevance of GPI-anchored proteins in the process of erythrocyte invasion, *B. bovis* isolated merozoites were treated with phospholipase C and then evaluated in their ability to invade erythrocytes. Determination of viability as outlined in Treatment of *B. bovis* merozoites with phospholipase C section showed a high survival rate of non-treated merozoites (97.6%; 1378 of a total of 1412 counted merozoites were determined as viable) and also of PI-PLC-treated merozoites (96.8%; 692 of a total of 715 counted merozoites were determined as viable). Based on Fisher's exact test ($p = 0.3196$), the null hypothesis stating "no change of viability observed between PI-PLC-treated and untreated merozoites" could not be rejected. It is noteworthy, that our mild procedure of releasing merozoites from infected erythrocytes in the cold seems to have resulted in a considerably higher viability (97.6%) than the possibly harsher high-voltage pulse treatment of infected erythrocytes as applied by Franssen et al. (2003) which resulted in a viability of over 80%.

PI-PLC-treated and non-treated merozoites were incubated with bovine erythrocytes in a ratio of motile merozoites to fresh erythrocytes of 1:1. For each cellular suspension, the increase of infected erythrocytes due to merozoite invasion was independently determined by microscopic examination of Giemsa-stained smears. In non-treated merozoite-erythrocyte suspensions, an IE increase (ΔIE) of 1.80% was observed at 4 h. An extended culturing of suspensions for up to 18 h resulted in a slightly increased ΔIE of 2.00% suggesting that merozoites might just have started to duplicate and reinvade erythrocytes (Fig. 1). In contrast, PI-PLC-treated merozoites/erythrocyte suspensions did not show any significant increase of ΔIE neither at 4 h nor after prolonged culturing for up to 18 h demonstrating a remarkable inhibition of invasion. Although Franssen et al. (2003) used a different parasite strain and merozoite release protocol (see above), they report an invasion rate in a similar range of ΔIE between 1.6% and 2.0% at 2 h and 4 h when using an infection ratio of 1 merozoite to 2 erythrocytes and/or of 1 merozoite to 10 erythrocytes.

This finding strongly suggests that GPI-anchored proteins play a crucial role in erythrocyte invasion and provides an explanation for the observation made by Rodriguez et al. (2010) that mannosamine-mediated inhibition of GPI synthesis impaired merozoite growth. It is furthermore in agreement with earlier reports showing that incubation of merozoites with specific antibodies against the *B. bovis* GPI-anchored proteins MSA-1, MSA-2a, MSA-2b, MSA-2a/b, and MSA-2c in each case substantially decreases their invasion capacity (Hines et al., 1992, 1995; Mosqueda et al., 2002a; Suarez et al., 2000; Wilkowsky et al., 2003). In this context, it is important to note that also a number of non-GPI-anchored *B. bovis* antigens like AMA-1 (Gaffar et al., 2004b), TRAP-like antigen (Gaffar et al., 2004a), RAP-1 (Mosqueda et al., 2002b), RAP-1-related antigen (RRA) (Suarez et al., 2011), a MIC-1-like antigen (Silva et al., 2010b), and Bbo-6cys-E (Silva et al., 2010a) have also been reported to neutralize erythrocyte invasion. It will be essential to reveal in the future how these proteins interrelate in the invasion process, and whether alternative invasion pathways exist as has been demonstrated for *Plasmodium* spp. (Dolan et al., 1990; Jiang et al., 2010; Silvie et al., 2007).

The enzymatic treatment of merozoites might have also acted on the abundant PI-PLC-sensitive GPI molecules not associated to proteins that are present in *B. bovis* membranes, as recently reported (Rodriguez et al., 2010). The effect of protein-free GPIs in *B. bovis* merozoite invasion of erythrocytes awaits further experimental examination.

In silico identification of GPI-anchored proteins

The entire *B. bovis* proteome containing 3706 proteins was explored for the existence of GPI-anchored proteins using several bioinformatic tools (Table 1). To this end, a search strategy was conceived to screen in 3 consecutive steps for the presence of the following shared features of GPI-anchored proteins: (i) a signal peptide, (ii) a GPI anchor-site sequence, and (iii) a C-terminal transmembrane domain. The sorting signal channels the protein into the ER and is an essential prerequisite for GPI-anchor attachment as exclusively the ER-resident transamidase can transfer the GPI moiety to the proprotein (Ferguson et al., 2009). SignalP 4.1 was used as it has been reported to predict the existence of an N-terminal signal with a very high sensitivity (98.6%) and an extremely low rate of false positives (1.2%) (Emanuelsson et al., 2007). Altogether,

Table 1
Proven and predicted GPI-anchored proteins of *Babesia bovis*.

	Gene	Functional annotation	Signal peptide	GPI anchor site				C-terminal TM	Length (aa)
			SignalP	bigPI[a]	GPI-SOM	Pred-GPI	FragAnchor	Topcons/DAS[b]	
1	BBOV_I002220A	hyp. prot.	+	+	+	HP	HP	+	657
2	BBOV_III000650	hyp. prot.	+	+	+	HP	P	+	1323
3	BBOV_II000410	hyp. prot.	+	+	+	HP	WP	+	293
4	BBOV_I003060A	MSA-1	+	+	+	HP	PFP	+	319
5	BBOV_I003020A	MSA-2c	+	+	+	HP	PFP	+	265
6	BBOV_I002990	MSA-2b	+	+	+	HP	PFP	+	316
7	BBOV_I003000	MSA-2a2	+	+	+	HP	PFP	+	316
8	BBOV_I003010A	MSA-2a1	+	+	+	HP	PFP	+	287
9	BBOV_III003710	hyp. prot.	+	+	+	HP	PFP	+	462
10	BBOV_IV002470	hyp. prot.	+	+	+	0	P	+	1016
11	BBOV_III004180	hyp. prot.	+	+	+	HP	P	+	387
12	BBOV_III004950	hyp. prot.	+	+	+	0	PFP	+	880
13	BBOV_I003130	hyp. prot.	+	0	+	HP	P	+	376
14	BBOV_IV010470	hyp. prot.	+	0	+	HP	PFP	+	479
15	BBOV_I001980	hyp. prot.	+	0	+	P	P	+	179
16	BBOV_IV006820A	hyp. prot.	+	0	+	P	PFP	+	170
17	BBOV_III004160A	hyp. prot.	+	0	+	WP	PFP	+	826

+, predicted; 0, not predicted; HP, high probability; P, medium probability; LP, low probability, PFP, possible false-positive.
[a] Predicted by any of the big-PI tools.
[b] Predicted by Topcons and/or DAS.

350 proteins were shown to contain a signal peptide and therefore further considered as possible GPI-anchored proteins.

GPI anchor-site sequences are highly complex structures, and the precision of prediction suffers limitations depending on the design of each bioinformatic tool. Existing tools are based on Hidden Markov Models (HMM), and the quality of prediction depends on their training with experimentally verified GPI anchor attachment sites which are often scarce. An additional complication is that the structure of GPI anchor attachment sites varies between different taxonomic classes and notably between protozoans which are an extremely divergent group from an evolutionary point of view. Based on this rationale, GPI anchor and other prediction tools were combined to enhance the predictive power in a manual voting approach. Voting approaches are increasingly applied and integrated in recent bioinformatic prediction tools, and they have been regularly shown to substantially increase the quality of predictions. GPI-SOM and big-PI GPI-anchored prediction tools make definite "yes/no" predictions. In contrast, Pred-GPI and FragAnchor assigns the following probability values to each prediction: HP (highly probable); P (probable), and LP (lowly probable). In addition, FragAnchor also assigns the probability criterion PFP (potentially false-positive). big-PI features a highly conservative prediction thus avoiding false positives, while accepting false-negative reports (corresponding to a prediction with a high specificity and low sensitivity). GPI-SOM, Pred-GPI, and FragAnchor predict with differing grades of permissiveness and include potentially false-positive hits (corresponding to a prediction of low specificity and high sensitivity). Therefore, following conservative selection criteria, a protein was accepted as being GPI-anchored when it was predicted by at least 3 of the 4 tools applied. Proproteins destined to be GPI-anchored include a C-terminal alpha-helix transmembrane region for membrane anchoring before the transfer of the protein to the GPI-anchor takes place (Ferguson et al., 2009). The TOPCONS and the DAS tool were used to verify the existence of a C-terminal transmembrane region in the predicted GPI-anchored proteins. All identified GPI-anchored proteins were found to contain a C-terminal transmembrane region by at least one of the 2 transmembrane prediction tools and therefore finally considered to represent GPI-anchored proteins (Table 1). These 17 proteins correspond to 0.4% of genome-encoded proteins of *B. bovis*, which falls within the previously estimated range for other pathogenic protozoans (Eisenhaber et al., 2001). In a recent work, a significant higher number of 73 putative GPI-anchored proteins have been reported using a single GPI anchor site-predictive tool (DGPI; Gohil et al., 2013). The difference is due to the use, in our work, of 4 GPI anchor site-predictive tools in combination with the outlined selection criteria and an imperative presence of a C-terminal transmembrane region. It is noteworthy that the previously characterized VMSAs that have been either experimentally shown (MSA-1), or strongly suggested (MSA-2a$_1$, MSA-2a$_2$, MSA-2b, MSA-2c), to contain a GPI anchor, are included in the final list validating the outlined approach (Carcy et al., 2006; Hines et al., 1989; Florin-Christensen et al., 2002; Suarez et al., 2000). All other identified putative GPI-anchored proteins have not been studied so far, and their patterns of expression and functions are unknown. They therefore represent prominent candidates for functional studies and for inclusion in subunit vaccines.

Occurrence and characterization of amino acid repeats in proven and predicted GPI-anchored proteins

GPI-anchored proteins are ultimately transported to the cell surface and are regularly involved in molecular mechanisms of recognition, adhesion, and transport (Ferguson et al., 2009). On a structural basis, these functions usually correspond with repetitive units in the primary sequence. Fifteen of the 17 identified GPI-anchored proteins contained amino acid repeats as assessed by RADAR. The detected repeats vary in length between 10 and 62 residues (Supplementary Table S1). The number of different repeat motifs varied from 1 to 8 in a single protein, and each was found to be 2–6 times repeated with sequence variations. In one case, altogether 26 repeat sequences were displayed in a single protein (encoding gene: BBOVIII000650), amounting for 50% of its length.

Repeat motifs are supposed to contain amino acids that promote the formation of epitopes and thus, antibody recognition. In view of the importance of polar residues in antibody–antigen binding, it has been put forward by Verra and Hughes (1999) that amino acid types might have a non-random occurrence in these regions. In order to determine in which way the amino acid composition is altered in repetitive vs. non-repetitive regions of *B. bovis* predicted GPI-anchored antigens, a chi-square test was carried out (Table 2). After correction by the number of statistical tests, proline, an amino acid known to strongly favor the formation of unstructured regions, was found to be highly significantly increased. It is well established that epitopes are frequently situated in unstructured regions. This finding is in line with a recently reported increased presence of

Table 2
Amino acid frequency in repeats compared to non-repeat sequences.

Amino acids	Non-repetitive sequences (%)	Repetitive sequences (%)	χ^2-test (p)
Alanine (A)	6.9	8.1	ns
Arginine (R)	2.9	3.5	ns
Asparagine (N)	5.3	5.5	ns
Aspartic acid (D)	7.9	5.6	***
Cysteine (C)	0.8	1.7	*
Glutamine (Q)	5.5	3.6	***
Glutamic acid (E)	7.8	6.2	***
Glycine (G)	5.1	4.9	ns
Histidine (H)	2.5	2.3	ns
Isoleucine (I)	5.5	6.1	ns
Leucine (L)	7.4	9.2	ns
Lysine (K)	6.4	6.7	ns
Methionine (M)	2.1	2.6	ns
Phenylalanine (F)	2.7	3.9	ns
Proline (P)	7.7	4.7	***
Serine (S)	8.8	8.0	ns
Threonine (T)	7.0	6.5	ns
Tryptophan (W)	0.3	0.6	ns
Tyrosine (Y)	2.4	3.2	ns
Valine (V)	5.1	7.0	*

p gives the level of significance after Bonferroni correction: ns, non-significant; * significant ($p \leq 0.05$); *** extremely significant ($p \leq 0.001$).

proline in repeats located in the hypervariable region of MSA-1 and MSA-2 antigens when allelic variants were compared (Berens et al., 2005; LeRoith et al., 2006). Among hydrophilic amino acids, aspartic acid, glutamic acid, and glutamine exhibited a highly significantly elevated frequency. Among hydrophobic amino acids, valine and also cysteine were found to be significantly decreased in repetitive regions. The latter observation is in accordance with a report that hydrophobic amino acids are underrepresented in repetitive regions as compared to non-repetitive regions in *Plasmodium* spp. (Verra and Hughes, 1999).

The above observation was further underlined by determination of the GRAVY value, which allows an estimation of the average hydrophobicity of different sequence regions (Kyte and Doolittle, 1982). Repeat regions (GRAVY value = −0.678) were found to be 3.21 times less hydrophobic than non-repeat regions (GRAVY value = −0.211).

Repeat regions in surface antigens have also been implicated in hemoparasite evasion of the host immune defense ("smoke screen hypothesis") (Kemp et al., 1987; Enea and Arnot, 1988; Bickle et al., 1993; Reeder and Brown, 1996). Certainly, those antigens that would be involved in immune evasion do not represent primary vaccine candidates. However, so far experimental evidence supporting the smoke screen hypothesis has neither been presented for *B. bovis* nor for any other hemoparasite surface antigens. Therefore, in any case, experimental work is needed to test the suitability of the newly highlighted *B. bovis* antigens in vaccine formulations.

Genomic localization and paralogous relationships of predicted GPI-anchored protein-encoding genes

The localization of putative GPI-anchored protein-encoding genes across the *B. bovis* genome was assessed. Eight GPI-anchored proteins were found to be encoded in chr I, one in chr II, 5 in chr III, and 3 in chr IV. Exclusively the gene family encoding the VMSA is organized in a single syntenic block of chr I. None of the remaining putative GPI-anchored protein-encoding genes are colocalized or exhibit paralogous relationships with each other as assessed by a reverse BLASTp test. However, putative GPI-anchored proteins encoded by gene loci BBOV_II000410 and BBOV_III004160 are each part of a gene family that includes 3 and 4 members of other predicted non-GPI-anchored proteins, respectively. Notably, also BBOV_III004160 is organized in a single syntenic block with its paralogous non-GPI-anchored relatives (Table 3). This exemplifies that phylogenetic relation does not necessarily correspond with the existence of a GPI anchor attachment signal as is observed for the *vmsa* family of genes.

Table 3
Gene families including GPI-anchored proteins in *B. bovis*.

No.	Family	Proteins in the family
1	VMSA family	**BBOV_I003010A, BBOV_I003000, BBOV_I002990, BBOV_I003020A, BBOV_I003060A**
2	Gene family A	**BBOV_II000410,** BBOV_IV005650, BBOV_I001050, BBOV_I001060
3	Gene family B	**BBOV_III004160,** BBOV_III004140A, BBOV_III004130, BBOV_III004200A, BBOV_III004190

Genes in bold encode GPI-anchored proteins; gene designations printed in non-bold letters encode non-GPI-anchored paralogs. Gene families have been identified by a reverse BLASTp test and synteny studies.

In summary, our study reveals the fundamental functional importance of GPI-anchored surface proteins for erythrocyte invasion and identifies previously unknown putative vaccine candidates that deserve more profound functional studies.

Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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References

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340, 783–795.

Berens, S.J., Brayton, K.A., Molloy, J.B., Bock, R.E., Lew, A.E., McElwain, T.F., 2005. Merozoite surface antigen 2 proteins of *Babesia bovis* vaccine breakthrough isolates contain a unique hypervariable region composed of degenerate repeats. Infect Immun 73, 7180–7189.

Bernsel, A., Viklund, H., Hennerdal, A., 2009. TOPCONS: consensus prediction of membrane protein topology. Nucleic Acids Res 37 (Webserver issue), W465–W468.

Bickle, Q., Anders, R.F., Day, K., Coppel, R.L., 1993. The S-antigen of *Plasmodium falciparum*: repertoire and origin of diversity. Mol Biochem Parasitol 61, 189–196.

Bock, R., Jackson, L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129 (Suppl.), S247–S269.

Brayton, K.A., Lau, A.O., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S.J., Bidwell, S.L., Brown, W.C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H.A., Haas, B.J., Howell, J.M., Khouri, H., Koo, H., Mann, D.J., Norimine, J., Paulsen, I.T., Radune, D., Ren, Q., Smith Jr., R.K., Suarez, C.E., White, O., Wortman, J.R., Knowles Jr., D.P., McElwain, T.F., Nene, V.M., 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathogens 3, 1401–1413.

Brown, W.C., Norimine, J., Goff, W.L., Suarez, C.E., McElwain, T.F., 2006. Prospects for recombinant vaccines against *Babesia bovis* and related parasites. Parasite Immunology 28, 315–327.

Caetano, B.C., Bruna-Romero, O., Fux, B., Mendes, E.A., Penido, M.L., Gazzinelli, R.T., 2006. Vaccination with replication-deficient recombinant adenoviruses

encoding the main surface antigens of *Toxoplasma gondii* induces immune response and protection against infection in mice. Hum Gene Ther 17, 415–426.
Carcy, B., Precigout, E., Schetters, T., Gorenflot, A., 2006. Genetic basis for GPI-anchor merozoite surface antigen polymorphism of *Babesia* and resulting antigenic diversity. Vet Parasitol 138, 33–49.
Coonrod, S.A., Naaby-Hansen, S., Shetty, J., Shibahara, H., Chen, M., White, J.M., Herr, J.C., 1999. Treatment of mouse oocytes with PI-PLC releases 70-kDa (pI 5) and 35- to 45-kDa (pI 5.5) protein clusters from the egg surface and inhibits sperm-oolemma binding and fusion. Dev Biol 207, 334–349.
Cserzo, M., Wallin, E., Simon, I., von Heijne, G., Elofsson, A., 1997. Prediction of transmembrane alpha-helices in procariotic membrane proteins: the Dense Alignment Surface method. Prot Eng 10, 673–676.
Dolan, S.A., Miller, L.H., Wellems, T.E., 1990. Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*. J Clin Invest 86, 618–624.
Eisenhaber, B., Bork, P., Eisenhaber, F., 2001. Post-translational GPI lipid anchor modification of proteins in kingdoms of life: analysis of protein sequence data from complete genomes. Protein Eng 14, 17–25.
Eisenhaber, B., Schneider, G., Wildpaner, M., Eisenhaber, F., 2004. A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. J Mol Biol 337, 243–253.
Emanuelsson, O., Brunak, S., von Heijne, G., Nielsen, H., 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2, 953–971.
Enea, V., Arnot, D., 1988. The circumsporozoite gene in *Plasmodium*. In: Turner, M., Arnot, D. (Eds.), Current Communications in Molecular Biology: Molecular Genetics of Parasitic Protozoa. Cold Spring Harbor Laboratory Press, New York, pp. 5–11.
Fankhauser, N., Maser, P., 2005. Identification of GPI anchor attachment signals by a Kohonen self-organizing map. Bioinformatics 21, 1846–1852.
Ferguson, M.A., 1999. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J Cell Sci 112 (Pt 17), 2799–2809.
Ferguson, M.A.J., Kinoshita, T., Hart, G.W., 2009. Glycosylphosphatidylinositol anchors. In: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanely, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E. (Eds.), Essentials of Glycobiology. Cold Spring Harbor Laboratory Press, New York, pp. 131–143.
Florin-Christensen, M., Suarez, C.E., Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., 2002. The *Babesia bovis* merozoite surface antigen 2 locus contains four tandemly arranged and expressed genes encoding immunologically distinct proteins. Infection and Immunity 70, 3566–3575.
Franssen, F.F., Gaffar, F.R., Yatsuda, A.P., de Vries, E., 2003. Characterisation of erythrocyte invasion by *Babesia bovis* merozoites efficiently released from their host cell after high-voltage pulsing. Microbes Infect 5, 365–372.
Gaffar, F.R., Yatsuda, A.P., Franssen, F.F., de Vries, E., 2004a. A *Babesia bovis* merozoite protein with a domain architecture highly similar to the thrombospondin-related anonymous protein (TRAP) present in *Plasmodium* sporozoites. Mol Biochem Parasitol 136, 25–34.
Gaffar, F.R., Yatsuda, A.P., Franssen, F.F., de Vries, E., 2004b. Erythrocyte invasion by *Babesia bovis* merozoites is inhibited by polyclonal antisera directed against peptides derived from a homologue of *Plasmodium falciparum* apical membrane antigen 1. Infection and Immunity 72, 2947–2955.
Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy server. In: Walker, J.M. (Ed.), The Proteomics Protocols Handbook. Humana Press, New York, pp. 571–607.
Gohil, S., Kats, L.M., Seemann, T., Fernandez, K.M., Siddiqui, G., Cooke, B.M., 2013. Bioinformatic prediction of the exportome of *Babesia bovis* and identification of novel proteins in parasite-infected red blood cells. Int J Parasitol 43, 409–416.
Hadj-Kaddour, K., Carcy, B., Vallet, A., Randazzo, S., Delbecq, S., Kleuskens, J., Schetters, T., Gorenflot, A., Precigout, E., 2007. Recombinant protein Bd37 protected gerbils against heterologous challenges with isolates of *Babesia divergens* polymorphic for the bd37 gene. Parasitology 134, 187–196.
Heger, A., Holm, L., 2000. Rapid automatic detection and alignment of repeats in protein sequences. Proteins 41, 224–237.
Hines, S.A., McElwain, T.F., Buening, G.M., Palmer, G.H., 1989. Molecular characterization of *Babesia bovis* merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol Biochem Parasitol 37, 1–9.
Hines, S.A., Palmer, G.H., Jasmer, D.P., Goff, W.L., McElwain, T.F., 1995. Immunization of cattle with recombinant *Babesia bovis* merozoite surface antigen-1. Infection and Immunity 63, 349–352.
Hines, S.A., Palmer, G.H., Jasmer, D.P., McGuire, T.C., McElwain, T.F., 1992. Neutralization-sensitive merozoite surface antigens of *Babesia bovis* encoded by members of a polymorphic gene family. Mol Biochem Parasitol 55, 85–94.
Holder, A.A., Freeman, R.R., 1981. Immunization against blood-stage rodent malaria using purified parasite antigens. Nature 294, 361–364.
Jiang, L., Lopez-Barragan, M.J., Jiang, H., Mu, J., Gaur, D., Zhao, K., Felsenfeld, G., Miller, L.H., 2010. Epigenetic control of the variable expression of a *Plasmodium falciparum* receptor protein for erythrocyte invasion. Proc Natl Acad Sci USA 107, 2224–2229.
Kemp, D.J., Coppel, R.L., Anders, R.F., 1987. Repetitive proteins and genes of malaria. Annu Rev Microbiol 41, 181–208.
Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J Mol Biol 157, 105–132.
LeRoith, T., Berens, S.J., Brayton, K.A., Hines, S.A., Brown, W.C., Norimine, J., McElwain, T.F., 2006. The *Babesia bovis* merozoite surface antigen 1 hypervariable region induces surface-reactive antibodies that block merozoite invasion. Infect Immun 74, 3663–3667.
Levy, M.G., Ristic, M., 1980. *Babesia bovis*: continuous cultivation in a microaerophilous stationary phase culture. Science 207, 1218–1220.
Low, M.G., 1989. Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. FASEB J 3, 1600–1608.
McConville, M.J., Ferguson, M.A., 1993. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 294 (Pt 2), 305–324.
Mosqueda, J., McElwain, T.F., Palmer, G.H., 2002a. *Babesia bovis* merozoite surface antigen 2 proteins are expressed on the merozoite and sporozoite surface, and specific antibodies inhibit attachment and invasion of erythrocytes. Infection and Immunity 70, 6448–6455.
Mosqueda, J., McElwain, T.F., Stiller, D., Palmer, G.H., 2002b. *Babesia bovis* merozoite surface antigen 1 and rhoptry-associated protein 1 are expressed in sporozoites, and specific antibodies inhibit sporozoite attachment to erythrocytes. Infection and Immunity 70, 1599–1603.
Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods 8, 785–786.
Pierleoni, A., Martelli, P.L., Casadio, R., 2008. PredGPI: a GPI-anchor predictor. BMC Bioinformatics 9, 392.
Plassmeyer, M.L., Reiter, K., Shimp Jr., R.L., Kotova, S., Smith, P.D., Hurt, D.E., House, B., Zou, X., Zhang, Y., Hickman, M., Uchime, O., Herrera, R., Nguyen, V., Glen, J., Lebowitz, J., Jin, A.J., Miller, L.H., MacDonald, N.J., Wu, Y., Narum, D.L., 2009. Structure of the *Plasmodium falciparum* circumsporozoite protein, a leading malaria vaccine candidate. The Journal of Biological Chemistry 284, 26951–26963.
Poisson, G., Chauve, C., Chen, X., Bergeron, A., 2007. FragAnchor: a large-scale predictor of glycosylphosphatidylinositol anchors in eukaryote protein sequences by qualitative scoring. Genomics Proteomics Bioinformatics 5, 121–130.
Reece, W.H., Pinder, M., Gothard, P.K., Milligan, P., Bojang, K., Doherty, T., Plebanski, M., Akinwunmi, P., Everaere, S., Watkins, K.R., Voss, G., Tornieporth, N., Alloueche, A., Greenwood, B.M., Kester, K.E., McAdam, K.P., Cohen, J., Hill, A.V., 2004. A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. Nature Medicine 10, 406–410.
Reeder, J.C., Brown, G.V., 1996. Antigenic variation and immune evasion in *Plasmodium falciparum* malaria. Immunol Cell Biol 74, 546–554.
Robinson, P.J., 1997. Signal transduction via GPI-anchored membrane proteins. Adv Exp Med Biol 419, 365–370.
Robinson, P.J., Millrain, M., Antoniou, J., Simpson, E., Mellor, A.L., 1989. A glycophospholipid anchor is required for Qa-2-mediated T cell activation. Nature 342, 85–87.
Rodriguez, A.E., Couto, A., Echaide, I., Schnittger, L., Florin-Christensen, M., 2010. *Babesia bovis* contains an abundant parasite-specific protein-free glycerophosphatidylinositol and the genes predicted for its assembly. Vet Parasitol 167, 227–235.
Rodriguez, S.D., Buening, G.M., Vega, C.A., Carson, C.A., 1986. *Babesia bovis*: purification and concentration of merozoites and infected bovine erythrocytes. Exp Parasitol 61, 236–243.
Silva, M.G., Ueti, M.W., Norimine, J., Florin-Christensen, M., Bastos, R.G., Goff, W.L., Brown, W.C., Oliva, A., Suarez, C.E., 2010a. *Babesia bovis* expresses a neutralization-sensitive antigen that contains a microneme adhesive repeat (MAR) domain. Parasitol Int 59, 294–297.
Silva, M.G., Ueti, M.W., Norimine, J., Florin-Christensen, M., Bastos, R.G., Goff, W.L., Brown, W.C., Oliva, A., Suarez, C.E., 2010b. *Babesia bovis* expresses Bbo-6cys-E, a member of a novel gene family that is homologous to the 6-cys family of *Plasmodium*. Parasitol Int 60, 13–18.
Silvie, O., Franetich, J.F., Boucheix, C., Rubinstein, E., Mazier, D., 2007. Alternative invasion pathways for *Plasmodium berghei* sporozoites. Int J Parasitol 37, 173–182.
Suarez, C.E., Florin-Christensen, M., Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., 2000. Characterization of allelic variation in the *Babesia bovis* merozoite surface antigen 1 (MSA-1) locus and identification of a cross-reactive inhibition-sensitive MSA-1 epitope. Infection and Immunity 68, 6865–6870.
Suarez, C.E., Laughery, J.M., Bastos, R.G., Johnson, W.C., Norimine, J., Asenzo, G., Brown, W.C., Florin-Christensen, M., Goff, W.L., 2011. A novel neutralization sensitive and subdominant RAP-1-related antigen (RRA) is expressed by *Babesia bovis* merozoites. Parasitology 18, 1–10.
Udenfriend, S., Kodukula, K., 1995. How glycosylphosphatidylinositol-anchored membrane proteins are made. Annu Rev Biochem 64, 563–591.
Verra, F., Hughes, A.L., 1999. Biased amino acid composition in repeat regions of *Plasmodium* antigens. Mol Biol Evol 16, 627–633.
Wilkowsky, S.E., Farber, M., Echaide, I., Torioni de Echaide, S., Zamorano, P.I., Dominguez, M., Suarez, C.E., Florin-Christensen, M., 2003. *Babesia bovis* merozoite surface protein-2c (MSA-2c) contains highly immunogenic, conserved B-cell epitopes that elicit neutralization-sensitive antibodies in cattle. Mol Biochem Parasitol 127, 133–141.