Babesia divergens: cloning and biochemical characterization of Bd37

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SUMMARY

The immunoprotective potential of *Babesia divergens* antigens released in supernatants of *in vitro* cultures of the parasite is generally known. Among a number of parasite molecules, a 37 kDa protein has been found in the supernatants of *Babesia divergens* cultures. In this report the cloning and biochemical characterization of this protein, called Bd37, are described†. In addition, the processing of the protein was studied *in vitro*. Results suggest that Bd37 is encoded by a single copy gene. Bd37 appears to be a merozoite-associated molecule attached to the surface by a glycosylphosphatidylinositol moiety containing a palmitate residue attached to the inositol ring. In addition, it is demonstrated that both extremities of the protein are linked by a disulphide bond. Results further indicate that a soluble, hydrophilic form of Bd37 can be released from the merozoite surface by GPI-specific phospholipase D. The potential role the Bd37 protein and the GPI anchor are discussed.

Key words: *Babesia divergens*, Apicomplexa, glycosylphosphatidylinositol, membrane-bound protein.

INTRODUCTION

Redwater in cattle is caused by infection with *Babesia divergens*; a protozoan parasite transmitted to bovines by the tick *Ixodes ricinus*. The parasites proliferate in the red blood cells of their hosts, leading to severe haemolytic anaemia in infected animals (Mehlhorn & Schein, 1984). Direct economic losses result from mortality and reduction in meat and milk production in infected cattle (Kuttler, 1988). Infected animals can be effectively cured with imidocarb dipropionate (Imidocarb®) but residues of this compound in the meat and milk lead to additional economic losses, as a withdrawal period has to be met. Control of redwater through vaccination would not have these drawbacks (Kuttler, 1988) and this is the reason why a vaccine-based research programme to search for immunoprotective babesial antigens is being developed.

It has been shown previously that cattle can be protected against *B. divergens* challenge infection using supernatants of *in vitro* cultures of the parasite (Valentin *et al*. 1993). A number of polypeptides of *B. divergens* are recognized by sera of recovered animals (Grande *et al*. 1998). Among these is a protein of 37 kDa which is a potentially membrane anchored protein, according to labelling experiments with [^{3}H]palmitate and [^{3}H]glucosamine (Carcy *et al*. 1995). The molecule (called Bd37) appears to be localized at the surface of merozoites and in vesicles inside the merozoite, and can be detected in the supernatant of cultures of *B. divergens*. In this paper we report the sequence and the biochemical characterization of Bd37.

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† The sequence data reported herein have been deposited in EMBL under Accession No. AJ422214.

MATERIALS AND METHODS

Animals

Female gerbils of 8 weeks old were obtained from Centre d'Elevage René Janvier (CERJ, Saint-Genest, France). Female SPF New Zealand rabbits were obtained from Elevage Scientifique des Dombes (France). All animals received feed and drinking water *ad libitum*.

In vitro *culture*

Babesia divergens strain Rouen 1987 was cultivated following a simplification of the method described by Gorenflot *et al*. (1991). Briefly, parasites were cultivated in RPMI 1640 (Life Technologies, France) supplemented with 25 mM HEPES and 10 mM sodium bicarbonate (pH 7·35), containing

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10 % human serum under 5 % CO_2 in air. Cultures were initiated at 1 % parasitaemia in a suspension of 5 % (v/v) human red blood cells (Centre de Transfusion Sanguine, Montpellier, France). When the parasitaemia reached 30–40 % parasites were subcultured by dilution with fresh red blood cells to 1 % parasitaemia.

Cloning of the gene coding the Bd37 protein and sequence analysis

A cDNA library from *B. divergens* strain Rouen 1987 was constructed in λZAP II (Stratagene, Netherlands). Total RNA was extracted from 2×10^9 parasitized red blood cells using RNAgents Total RNA Isolation System (Promega, France). Purified mRNA was obtained from total RNA using the PolyATract mRNA Isolation System III (Promega, France). The cDNA was synthesized using the ZAP Express cDNA synthesis kit (Stratagene, Netherlands). The cDNA library was packaged in viral particles by the Gigapack II packaging extract and this library was screened with a rabbit immune serum raised against the 37 kDa protein (Carcy *et al.* 1995). A positive clone was *in vivo*-excised using ExAssist Helper Phage (Stratagene) and the recombinant λZAP bacteriophage was converted in a recombinant phagemide vector which was sequenced by Genome Express S.A. (Meylan, France).

The nucleotide sequence was translated using Editseq (Lasergene Package, DNAstar, USA) and the deduced aminoacid sequence was submitted to PSI-BLAST at the NCBI website (http://www3.ncbi.nlm.nih.gov). N-terminus signal prediction was performed using SignalPserver (http://www.cbs.dtu.dk).

Cloning and expression of the recombinant His-Bd37

The core coding sequence of Bd37 (without N-terminus and C-terminus hydrophobic end-sequences) was amplified with *Pfu*Turbo polymerase (Stratagene) with the primers pQE-Bd37Up: 5′-AATGGCAATAAT**GGATCC**TGCACCAATCTC-3′ and pQE-Bd37Down: 5′-GAAGGATGGCTT**AAGCT T**ACTAGATCCCTG-3′. These primers introduce a *Bam*HI and *Hin*dIII restriction site used to insert the amplified sequence in the pQE-30 vector (Qiagen, France) in order to produce the His-Bd37 recombinant protein.

Recombinant pQE-30 His-Bd37 plasmid was transformed into *E. coli* M15[pREP4] (Qiagen) and expression of the His-Bd37 protein was carried out in LB medium as follows. Bacteria were grown at 37 °C and the expression of the recombinant protein was induced with 1 mM IPTG for 4 h. Cells were harvested and resuspended in lysis buffer (sodium phosphate buffer 20 mM, NaCl 500 mM, pH 7·4) containing 1 mg/ml of lysozyme and 2 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A and leupeptin. The cell suspension was frozen at −20 °C, thawed and incubated with DNAse I at 4 °C until the suspension was no longer viscous. The suspension was then centrifuged at 13000 ***g*** for 30 min at 4 °C and the supernatant was filtered through 0·22 μm filters. His-Bd37 was purified using a HiTrap chelating column charged with $NiSO_4$ on an FPLC apparatus (Amersham Pharmacia Biotech, France). The column was washed using lysis buffer containing 20 mM imidazole and by injection of lysis buffer containing 0·5 % of Triton X–100. His-Bd37 was eluted using lysis buffer containing 200 mM imidazole. The purified recombinant protein was desalted using a HiTrap desalting column equilibrated in phosphate-buffered saline (PBS, pH 7·4).

Nucleic acid hybridizations

Total RNA from 2×10^9 erythrocytes parasitized by *B. divergens* was extracted using RNAgents Total RNA Isolation System (Promega, France). RNA was subjected to electrophoresis in denaturing agarose gels and blotted as described (Sambrook, Fritsch & Maniatis, 1989).

Southern blot was performed using a standard procedure with genomic DNA extracted from *in vitro* culture using a Nucleospin Blood column (Macherey-Nagel, France) digested for 2 h with restriction enzymes. A fragment of the Bd37 gene was amplified from the recombinant pBK-CMV using the sense Bd37-3 primer (5′-TGATGAAGCCGGCAAGAAGGT-3′) and reverse Bd37-C primer (5′-AGAAGAAGGTACAGCAGCGAAGAA-3′) (Fig. 1). The fragment was then labelled with digoxigenin using the DIG-High Prime kit (Roche, France). Blots were probed and processed according to the manufacturer's instructions.

Genomic DNA from *B. divergens* (10 μg) was digested for 4 h at 37 °C by *Eco*RI, phenol-extracted and precipitated. The digested DNA was dissolved in 100 μl of ligation buffer 1 × (MBI Fermentas, France) containing 1 mM ATP and 3 U of T_4 DNA ligase (MBI Fermentas) and incubated overnight at 4 °C. Inverse PCR was then performed using primers Bd37-3 and Bd37-8 (5′-GCCTCACGCTGCCCCCTGAG-3′) (Fig. 1).

Metabolic labelling and immunoprecipitation

In vitro culture of *B. divergens* strain Rouen 1987 were initiated with 1 % parasitaemia. When parasitaemia reached 10 %, the culture medium was replaced with RPMI 1640 (Life Technologies) containing 50 μCi/ml of [^{3}H]ethanolamine (Amersham) or with RPMI 1640 without methionine (Life Technologies) and supplemented with 50 μCi/ml of

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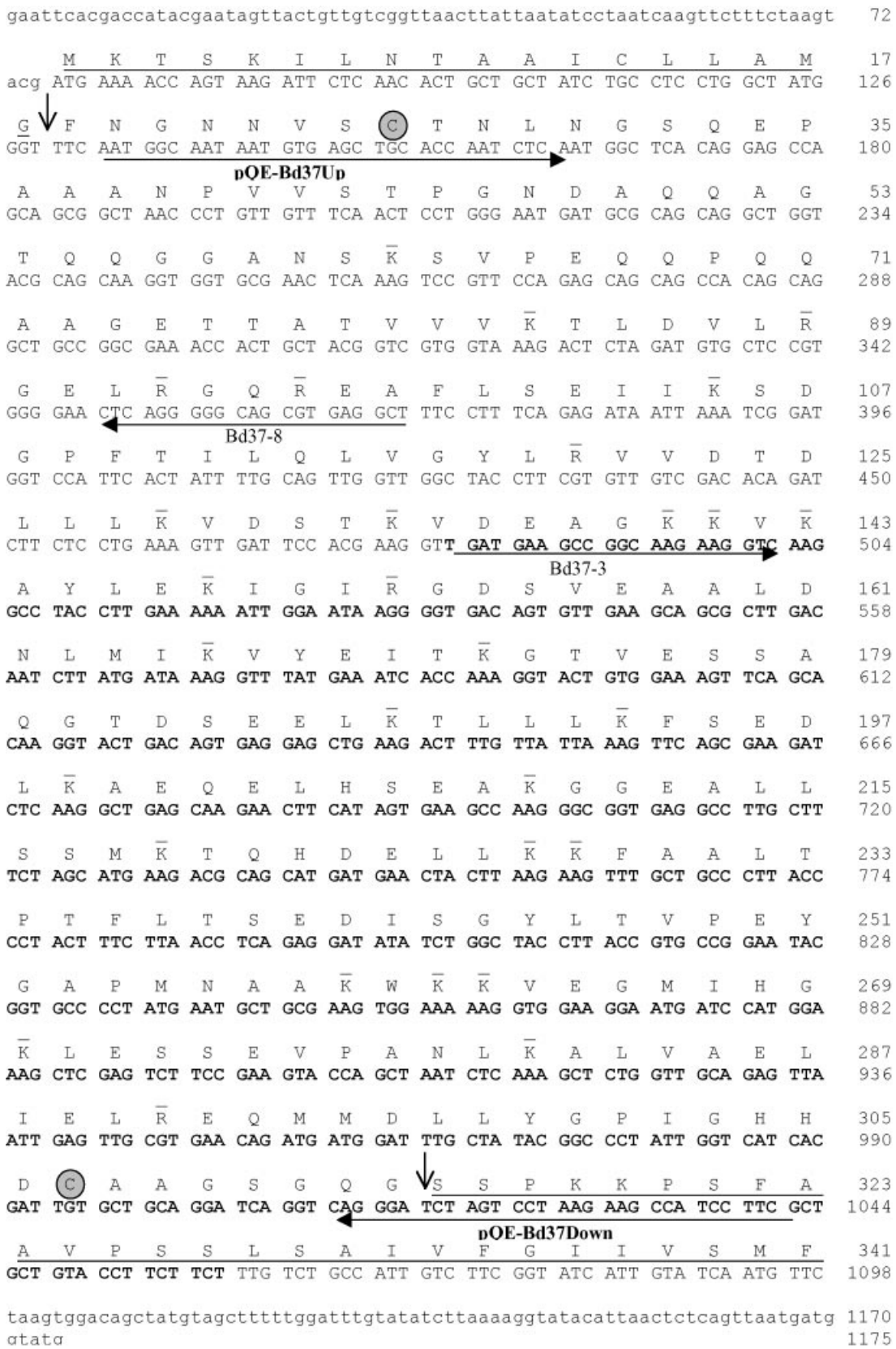

Fig. 1. Nucleotidic sequence of the cDNA encoding Bd37 and deduced amino acid sequence of the protein. Hydrophobic signal peptides are underlined, vertical arrows indicate predicted cleavage site. The primers used to clone in pQE-30 vector and to the inverse PCR are located under the sequence. The nucleotidic sequence bolded represents the DNA sequence used as probe for nucleic acid hybridizations. Cysteines are circled and ▼ are potential trypsin cleavage sites.

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[^{35}S]methionine (Amersham). Cultures were grown overnight as described above with 10% (v/v) human serum. The development of parasites was assessed by thin blood smears stained with Diff-Quick stain (Dade-Boehring, France).

The parasitized red blood cells were harvested by centrifugation and washed thrice in PBS. Packed cells were lysed in 10 volumes of RIPA buffer (KCl 0·6 M, NaCl 0·15 M, Tris–HCl 0·01 M, 2% Triton X–100, pH 7·8). Radio-isotope labelled lysate was incubated overnight at 4 °C after the addition of 7 μl of anti-Bd37 rabbit serum on a rocker platform. Subsequently, the lysate was incubated for 1 h with 75 μl of Protein A-Sepharose beads (Amersham Pharmacia Biotech) at room temperature while shaking. The immunoprecipitate was resolved by SDS–PAGE. Electrophoresis gels, after incubation in Amplify (Amersham, France) and drying, were autoradiographed with BioMax MR film (Kodak, France).

In vitro *translation of Bd37*

The Bd37 gene was transcribed and translated in rabbit reticulocyte lysate using the TnT T7 Quick Coupled Transcription/Translation System (Promega, France). PCR product containing T7 promoter and Ribosome Binding Site upstream to the Bd37 gene was used as template. The sense primer containing promoter and ribosome binding site was Bd37-T7 (5′-TAATACGACTCACTATAG-GAACAGACCACCATGAAAACCAGTAAGA-TTCTCAAC-3′) and the reverse primer was Bd37-Cterm (5′-CATACCATCATTAACTGAGA-3′). The coupled transcription and translation reaction was performed with and without addition of 2 μl of canine pancreatic microsomal membranes (Promega) according to the manufacturer's instructions. Reaction products were resolved on SDS–PAGE and gels were autoradiographed.

Temperature-induced phase partitioning using Triton X–114

This separation method was adapted from that reported by Bordier (1981). Parasitized erythrocytes were washed twice in TBS (Tris-buffered saline, Tris 10 mM, NaCl 150 mM, pH 8) and lysed in 10 volumes of TBS containing 1% (v/v) of Triton X–114. Lysates were incubated for 1 h on ice and centrifugated (13000 ***g***) at 4 °C. The TX–114 insoluble pellet was washed thrice in TBS with 1% TX–114 (v/v) at 4 °C. The supernatant fraction containing TX–114-soluble proteins was submitted to phase partitioning by incubation at 37 °C for 5 min followed by centrifugation. The detergent-enriched phase was resuspended in TBS at 4 °C. Then the aqueous and detergent-enriched phases were adjusted to 1% TX–114 and resubmitted to phase partitioning. This step was repeated 3 times for both phases. The detergent-enriched phase was precipitated by cold acetone and resuspended in TBS. Proteins from each phase were run on SDS–PAGE and subjected to Western blotting.

Enzymatic treatments

Cleavage of GPI anchors by glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) was performed in GPI-PLD buffer (Tris 50 mM, $CaCl_2$ 2·6 mM, Triton X–100 0·1% (v/v), pH 7·5). Parasitized erythrocytes (50 μl) were treated with 150 μl of GPI-PLD buffer and the lysate was divided over 2 tubes. One tube received 10% (v/v) of fresh gerbil serum as source of GPI-PLD and the other tube received gerbil sera and 5 mM of 1,10-phenanthroline as GPI-PLD inhibitor (Doerrler *et al*. 1996).

Cleavage of GPI anchor by phosphatidylinositol-specific phospholipase C (PI-PLC) was performed in TBS using PI-PLC from *Bacillus cereus* (Boehringer Mannheim, France) according to the method reported by Chen *et al*. (1998). Treatment by hydroxylamine was performed overnight on ice (1 M hydroxylamine in 50 mM triethanolamine, pH 10·7). The control sample was incubated overnight on ice in Tris 50 mM, pH 7·5. Samples were washed with TBS and concentrated on Centricon YM 3 kDa (Millipore, France).

Limited proteolysis

Recombinant His-Bd37 protein was incubated on ice in PBS with 25 μg/ml of TPCK-treated trypsin (Sigma). The digestion was stopped by addition of PMSF at 2 mM. Samples were then boiled in SDS–PAGE sample buffer with or without 50 mM β-mercaptoethanol and submitted to electrophoresis.

RESULTS

Sequence of Bd37

Upon screening of the cDNA library a λZAP clone expressing a β-galactosidase fusion protein was isolated, which was recognized by the anti-Bd37 immune serum. After the *in vivo* phage excision procedure a recombinant pBK-CMV plasmid containing the gene coding for Bd37 was isolated. Sequence analysis of the plasmid revealed that the pBK-CMV Bd37 contained a 1321 bp cDNA clone (Fig. 1). A single open reading frame (ORF) was found in the sequence. The deduced amino acid sequence corresponds to a protein with a predicted molecular weight around 37 kDa and an isoelectric point of 5·19. Sequence comparisons with BLAST or PSI-BLAST (Altschul *et al*. 1997) against non-

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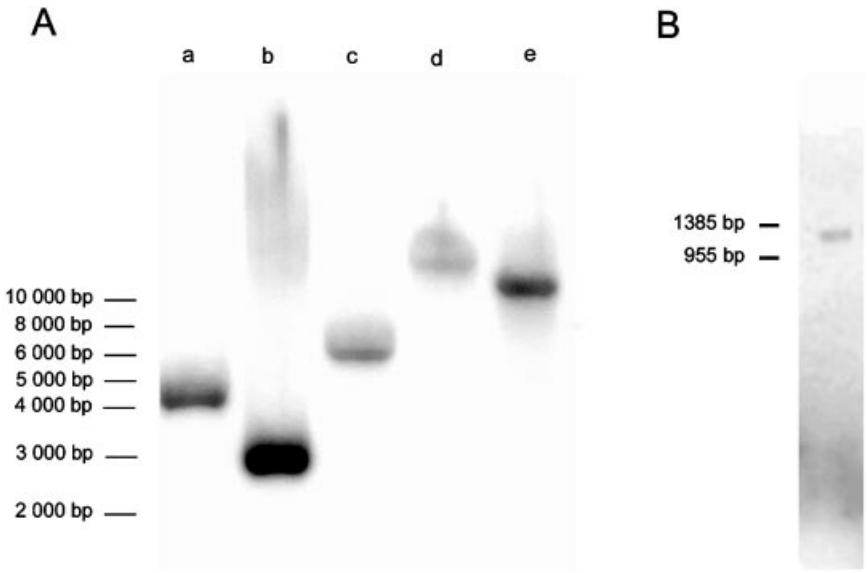

Fig. 2. Nucleic acid hybridization using Bd37 probe. (A) Southern blot with genomic DNA digested by: *Bam*HI (lane a), *Eco*RI (lane b), *Hin*dIII (lane c), *Kpn*I (lane d) and *Sma*I (lane e). (B) Northern blot performed using total RNA from *Babesia divergens*.

Fig. 3. *In vitro* processing of Bd37: lane 1: immunoprecipitation with antiBd37 serum performed on parasitic lysate metabolically labelled with [^{35}S]methionine, lane 2: *in vitro* translation of entire Bd37 cDNA in rabbit reticulocyte lysate, lane 3: *in vitro* translation of Bd37 cDNA in the presence of microsomal membrane, band A correspond to the complete protein and band B correspond to the processed protein.

redundant databases have given no significant homologies with described proteins. Hydrophobicity plots indicate the presence of hydrophobic residues at both the N-terminus and C-terminus (data not shown). These hydrophobic peptides corresponded to an N-terminal signal peptide with a predicted cleavage site between G^{18} and F^{19} (amino acid numerotation following Fig. 1) and to a C-terminal signal peptide for a glycosylphosphatidylinositol (GPI) anchor.

Bd37 is a single copy gene

Southern blot analysis with restriction enzymes not cutting within the coding sequence revealed a single band pattern (Fig. 2A, lanes a–e). To confirm the absence of tandemly repeated genes of Bd37, the genomic DNA was digested with *Eco*RI (producing a 3000 bp fragment containing the Bd37 gene). The genomic DNA fragments were ligated in diluted conditions to allow circularization of fragments. Then an inverse PCR was performed using Bd37 internal primers. The amplified fragment was sequenced and no additional ORF was found in this fragment (data not shown). These data lead to the conclusion that Bd37 is a single copy gene.

Northern blot analysis revealed a single transcript of about 1200 bp corresponding to the size of the cDNA (Fig. 2B). Moreover, the comparison of the Bd37 cDNA and PCR-amplified genomic Bd37 indicated that there are no introns in the sequence of Bd37 (data not shown).

The N-terminus signal peptide of Bd37 is cleaved in vitro

The product of the *in vitro* translation of the Bd37 gene migrated more slowly SDS–PAGE than the Bd37 protein immunoprecipitated from [^{35}S]-methionine labelled parasitic lysate (Fig. 3, lanes 1 and 2). The addition of microsomal membranes in the *in vitro* translation reaction induced the production of 2 bands (Fig. 3, lane 3, bands A and B). The upper band A corresponded to the protein produced *in vitro* without microsomal membranes and the lower band B corresponded to the protein immunoprecipitated from the parasitic lysate. The microsomal membranes were able to cleave signal peptides (manufacturer's information), so we conclude that the N-terminal signal peptide of Bd37 is cleaved.

Bd37 is anchored by GPI with an acylated inositol

Metabolic labelling of *B. divergens* in *in vitro* culture with [^{3}H]ethanolamine followed by immunoprecipitation using anti-Bd37 serum showed that Bd37 can

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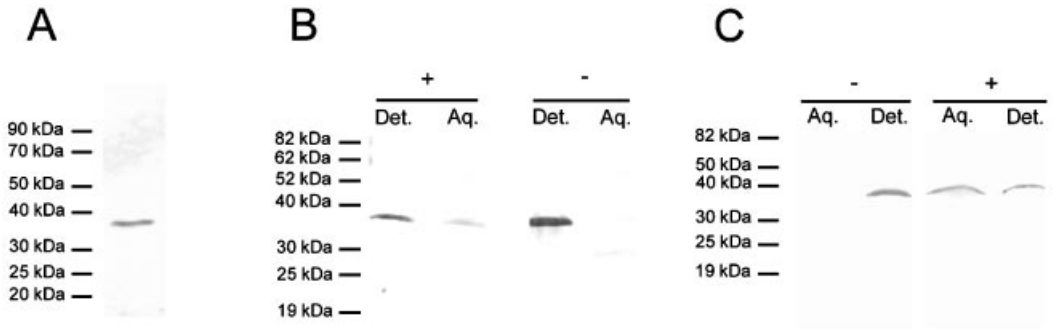

Fig. 4. Membrane anchorage of Bd37. (A) Immunoprecipitation with antiBd37 serum performed on parasitic lysate metabolically labelled with [^{3}H]ethanolamine. (B) Temperature-induced phase partitioning performed on GPI-PLD treated parasitic lysate without inhibitor (+) or with 1,10-phenanthrolin (−). Det., detergent-enriched phase; Aq., aqueous phase. (C) temperature-induced phase partitioning performed on PI-PLC treated parasitic lysate with (+) or without (−) hydroxylamine treatment.

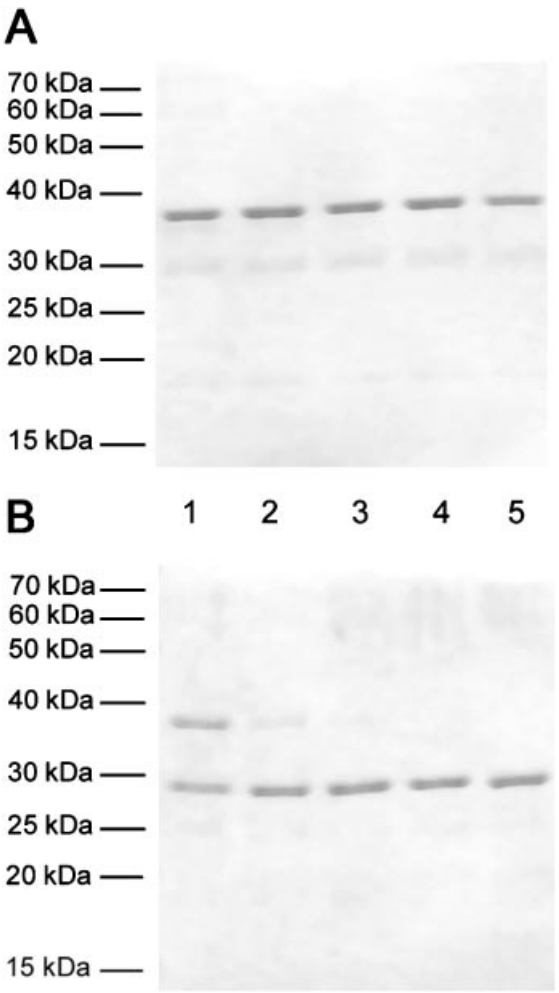

Fig. 5. Time-course of trypsin proteolysis of His-Bd37 electrophoresed in non-reducing conditions (A) and in reducing conditions (B). Lanes 1: 5 min, lane 2: 10 min, lane 3: 20 min, lane 4: 30 min, lane 5: 60 min.

be labelled with ethanolamine (Fig. 4A). As ethanolamine is an essential component of a GPI anchor, a positive labelling with ethanolamine indicates that the protein is potentially GPI anchored.

Additional experiments were performed to confirm anchoring of Bd37 by a GPI. The glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is able to cleave any GPI anchor leaving the protein part soluble. Upon GPI-PLD treatment of parasitic extracts Bd37 was detected in the aqueous phase of TX–114 temperature-induced phase partitioning (Fig. 4B, lanes +). In the presence of 1,10-phenanthroline no Bd37 was detected in the aqueous phase (Fig. 4B, lanes −). The data suggest that the anchor of Bd37 is specifically sensitive to GPI-PLD. This result and the fact that Bd37 can be labelled with ethanolamine (see above) demonstrated that Bd37 is a GPI-anchored protein.

Moreover, the PI-PLC from *Bacillus cereus* was unable to release Bd37 into the aqueous phase (Fig. 4C, lanes −). Resistance of a GPI anchor to PI-PLC could be the consequence of an acylation on the inositol ring in the carbohydrate part of the anchor. To de-acylate the inositol ring, the parasitic lysate was submitted to hydroxylamine treatment prior to PI-PLC digestion (Gowda *et al.* 1997). The sensitivity of the Bd37 anchor to PI-PLC was restored after treatment with hydroxylamine and Bd37 was released in the aqueous phase of the TX–114 experiment (Fig. 4C, lanes +). This last result indicates that the GPI anchor of Bd37 contains a fatty acid chain on the inositol of the GPI anchor.

Cysteines form a disulfide bond in Bd37

The recombinant His-Bd37 was expressed in *E. coli* as a soluble protein with a relatively high yield (50 mg/l). In order to assess the folding of the protein, we submitted His-Bd37 to proteolysis with trypsin. Although the sequence has 29 potential cleavage sites (Fig. 1), only a single fragment of 29 kDa was detected after digestion of His-Bd37 with trypsin (Fig. 5B). There was no further degradation of the 29 kDa fragment, even after digestion for 1 h (Fig. 5, lane 5) indicating that this remaining peptide is structured after the trypsin cleavage.

There are 2 cysteines (C^{26} and C^{307}) in the processed form of Bd37 located at each extremity of

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the protein (Fig. 1). The digestion product was electrophoretically separated in the presence and in the absence of β-mercaptoethanol (Fig. 5A and B). The apparent molecular weight of the undigested recombinant protein was the same as the trypsin digestion product electrophoresed in the absence of β-mercaptoethanol (Fig. 5A). This implies that the 2 cysteines are engaged in a disulfide bond holding the 2 tryptic fragments together. Moreover, this result suggests that the molecule is split into only 2 fragments, implying that trypsin has a single cleavage site accessible in the His-Bd37. The smaller fragment was a single 8 kDa fragment that was detected after the reducing electrophoresis (Fig. 5B). Most likely this fragment had run off the bottom of the gel. The resistance of the 2 generated fragments to further trypsin degradation indicated that these fragments were sufficiently structured to obscure the trypsin cleavage site. The position of the cysteines in the sequence suggests that the N-terminus and C-terminus of the protein are structurally closely associated in the folded protein.

DISCUSSION

An antigenic molecule of *B. divergens* with a relative molecular weight of 37 kDa (Bd37) can be detected at the surface of *B. divergens* merozoites (Carcy *et al.* 1995) and in the supernatant of *in vitro* cultures (Grande *et al.* 1998). The results presented here provide evidence that Bd37 is encoded by a single copy gene, and that it contains an N-terminal signal peptide. Furthermore, results suggest that Bd37 is attached to the merozoite membrane by a GPI-anchor. GPI anchorage has been described as the predominant surface protein anchorage pathway in parasitic protozoa such as *Plasmodium* (Gowda *et al.* 1997) and *Trypanosoma* (Ferguson *et al.* 1994) and appears to be the default pathway to target proteins to the surface of merozoites in Apicomplexa, in contrast to transmembrane proteins with a micronem or rhoptry cytoplasmic targeting signal (Ngo *et al.* 2000). The evidence that Bd37 is attached by a GPI anchor to the merozoite surface is 3-fold. First, Bd37 can be labelled with ethanolamine, which is an essential component of the GPI-anchor. Second, a soluble hydrophilic form of Bd37 could be recovered from the aqueous phase of TX–114 after incubation of parasite extracts with GPI-specific phospholipase D, while no such form was detected when the phospholipase D inhibitor 1,10-phenanthroline was added. Third, amino acid sequence data supported the presence of a GPI-anchor in Bd37.

Amino acid requirements for a GPI anchor are different in protozoa than in mammals (Moran & Caras, 1994). The parasitic transamidase needs a GPI cleavage/attachment site containing at the omega position (the last C-terminal residue after cleavage of the signal sequence) one of the following amino acids G, A, S, C, D, N and at the omega + 2 position either a G, A or S. The 3 residues composing the cleavage/attachment site must be followed by a stretch of 5 charged amino acids and/or a proline just before the hydrophobic sequence. This hydrophobic sequence starts at the F^{322} and stretches to the C-terminal amino acid end and there is a stretch of charged residues and prolines (PKKPS) just after a potential cleavage/attachment site (GSS). So we predict that G^{314} is the omega residue for the GPI anchor of the Bd37.

The merozoite membrane-bound form of Bd37 was previously shown to be palmitoylated in contrast to the non-palmitoylated soluble form observed in supernatants of *in vitro* culture. The resistance of the Bd37 GPI anchor to PI-PLC indicates an acylation of the inositol ring in the GPI anchor like that demonstrated for other parasites such as *Plasmodium* or *Trypanosoma*. This suggest that the GPI anchor of Bd37 contains an inositol ring acylated by a palmitate. The recently solved structure of a GPI anchor from *P. falciparum* indicates that 90% of fatty acid on the acylated inositol ring is a palmitate at the C-2 position (Naik *et al.* 2000). Hence, the structure of the *B. divergens* GPI anchor seems to be highly similar to the *P. falciparum* GPI anchor. The GPI structure of other Apicomplexa has not been precisely determined but we can suppose that *P. falciparum* GPI structure could be extended to other Apicomplexa.

The GPI-anchored surface molecules could play a role in the evasion of the host's immune response. The surface coat of *Trypanosoma* is made up of glycoproteins that are antigenically variable (Variable Surface Glycoprotein, VSG). These VSG molecules are attached to the surface by a GPI anchor, which allows rapid replacement of the entire cell surface coat and a rapid turnover of the different copies of VSG molecules (Donelson *et al.* 1998). In this way *Trypanosoma* is able to escape from specific immune responses targeted to the VSG molecules. Although it seems to have been demonstrated by pulse-chase experiments that, after its synthesis in the merozoite, Bd37 can be rapidly released into the *in vitro* culture supernatants (Grande *et al.* 1998), it is not likely that Bd37 of *B. divergens* has a similar role as VSG of *Trypanosoma*, because Bd37 exists as a single copy gene. This implies that *B. divergens* would not be able to produce a spectrum of antigenically different Bd37 molecules to avoid immune responses.

Alternatively, the GPI-anchored proteins at the surface of merozoites could be potentially involved in invasion mechanisms. It has been shown that the GPI-anchored glycoproteins of *Toxoplasma gondii* (Surface Antigens Glycoproteins; SAGs) attach to the host-cell surface (Grimwood & Smith, 1996). In addition, the Major Surface Protein 1 (MSP-1) of *Plasmodium falciparum*, which is also a GPI-

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anchored surface protein, binds to the host cell; the human erythrocyte (Nikodem & Davidson, 2000). It has been suggested that these surface molecules are potential targets for host-protective antibodies. Rapid release of soluble form of the molecules could serve to neutralize deleterious antibodies. Whether Bd37 of *B. divergens* is involved in host cell attachment and/or immune evasion remains to be determined.

Although Bd37 is found in the supernatants of *in vitro* cultures it is not known how it is released from the merozoites. It could be supposed that the protein is shed during erythrocyte invasion, cleaved off at the GPI anchor by phospholipase or released during merozoite lysis (Igarashi *et al.*1988). The fact that the GPI-PLD from mammalian sera is able to cleave the Bd37 GPI anchor suggests that the soluble form of Bd37 should be present in blood of *B. divergens*-infected animals. Whether this is the case, and what role soluble Bd37 could have in the host–parasite relationship is subject to further studies.

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