Identification and Characterization of the RouenBd1987 Babesia divergens Rhopty-Associated Protein 1

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Abstract

Human babesiosis is caused by one of several babesial species transmitted by ixodid ticks that have distinct geographical distributions based on the presence of competent animal hosts. The pathology of babesiosis, like malaria, is a consequence of the parasitaemia which develops through the cyclical replication of Babesia parasites in a patient’s red blood cells, though symptoms typically are nonspecific. We have identified the gene encoding Rhopty-Associated Protein –1 (RAP-1) from a human isolate of B. divergens, Rouen1987 and characterized its protein product at the molecular and cellular level. Consistent with other Babesia RAP-1 homologues, BdRAP-1 is expressed as a 46 kDa protein in the parasite rhoptries, suggesting a possible role in red cell invasion. Native BdRAP-1 binds to an unidentified red cell receptor(s) that appears to be non-sialylated and non-proteinacious in nature, but we do not find significant reduction in growth with anti-rRAP1 antibodies in vitro, highlighting the possibility the B. divergens is able to use alternative pathways for invasion, or there is an alternative, complementary, role for BdRAP-1 during the invasion process. As it is the parasite’s ability to recognize and then invade host cells which is central to clinical disease, characterising and understanding the role of Babesia-derived proteins involved in these steps are of great interest for the development of an effective prophylaxis.

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Introduction

The phylum Apicomplexa consists of protozoan parasites, among which are a number of etiological agents of medical and veterinary importance, including Plasmodium, Toxoplasma, Babesia, Neospora, Cryptosporidium, Eimeria and Theileria. They are defined by a common set of apically located secretory organelles that are required for host cell invasion [1–3]. Although the host cell range of Apicomplexan parasites is large and varied, the mechanism of host cell entry is thought to be conserved [4].

The genus Babesia comprises many species of parasites that invade RBCs of many different vertebrate hosts [3]. They are transmitted by their tick vectors during the taking of a blood meal from the vertebrate host [5,6]. In the last 50 years babesiosis has emerged as a major public health concern due to the expansion of the geographic range of the vectors, ixodid ticks [7,8]. Additionally, mortality rates average 30% to 45% in susceptible hosts, which have not been identified in any of the Babesia species. However a
Figure 1. Multiple sequence alignment of BdRAP-1 sequences shows homology to other babesial RAP-1 proteins is within the N-terminal portion. Clustal analysis of RAP-1 sequences of *B. divergens* (human host source) (accession number KJ699102) identified in this manuscript, *B. divergens* (bovine host origin) (accession number Z49818), *B. bovis* (accession number M38218) and *B. canis* (accession number M91168) shows that homology is restricted to the N-terminus region, which corresponds to the location of the RAP-1 superfamily structure (indication above the sequence by a sold line from BdRAP-1 Ala41 to Gly291) as identified by BLAST analysis. The sites of non-synonymous differences
number of rhoptry molecules classified as RAP-1 and RAP-2 have been characterized in some of the veterinary parasite species. There is a brief report on the gene sequence of a RAP-1 in B. divergens [19] isolated from a bovine source, although there are no further details on the characterization of this antigen. As B. divergens is an important human zoonosis, it is prudent to fully assess genes responsible for RBC invasion from parasites that result in human infection to aid the most effective interventions. We have identified and characterized a Rhopty Associated Protein —1 (RAP-1) homolog of BdRouen1987 isolated from a human infection, and provide additional support for this antigen role in invasion.

Materials and Methods

Animal Protocol Work and Ethics Statement

Cattle and gerbil sera were produced in 1995 under license in the Republic of Ireland (License number B100/702, Department of Health, Cruelty to Animal Act, 1876 (European Directive 86/609/EC) as part of studies on vaccination against bovine babesiosis. The license provided permitted the use of infesting with ticks, infecting with Babesia divergens by intraperitoneal injection, taking of blood spots from the tail for blood smears and bleeding while under halothane anaesthesia without recovery (euthanasia). As no formal ethics (IACUC) committees for animal experimentation existed in Ireland at the time (1995), the conditions of the licence were approved at the University College Dublin by both the Director of the Biomedical Facility (the departmental animal facility) and the Dean of the Faculty of Veterinary Sciences.

Briefly, yearling cattle housed on the University College Dublin farm were inoculated subcutaneously behind the left shoulder with 1 x 10^7 gerbil (Meriones unguiculatus) RBCs infected with the TL strain of B. divergens and suspended in 60% RPMI 1640 in HEPES with added L-glutamine and 40% fetal calf serum. Four weeks later approximately 100 mL of venous blood were taken by syringe from the jugular vein, and antibody levels of 1:256 titre in sera determined by IFA. The sera were then stored at −70°C.

Gerbils were housed at the Biomedical Facility, University College Dublin and sera were prepared by intraperitoneal inoculation of a group of 8-week old gerbils with PBS-suspended 1 x 10^7 erythrocytes infected with B. divergens (DR strain). Three weeks later the gerbils in which transient parasitaemia had been observed were challenged with 1 x 10^3 infected erythrocytes subdermally and then a further three weeks with 1 x 10^7 infected erythrocytes. After a further 4 weeks, all gerbils were bled by cardiac puncture under halothane anaesthesia and sacrificed after bleeding by intraperitoneal injection of sodium pentobarbitone formulated for euthanasia, and the resulting sera stored at −70°C.

Babesia divergens strain BdRouen1987 was first isolated from a French patient in 1986 [20] and propagated in culture, and has been used by many laboratories as the reference strain for B. divergens studies [21–25].

Parasite propagation

Asexual erythrocytic cultures of B. divergens (BdRouen1987 isolated from a French patient) [20] were maintained in vitro in human A* blood using RPMI 1640 medium (Life Technologies Corporation, Carlsbad, CA) supplemented with 10% human serum and sodium bicarbonate solution 7.5% (w/v) (Life Technologies Corporation, Carlsbad, CA). Cells were cultured at 37°C in 90% CO2, 5% nitrogen and 5% oxygen.

Identification and confirmation of the cDNA and gDNA Bdrap-1 sequences

The Bdrap-1 sequence was obtained by initially screening a B. divergens cDNA library [22] by PCR with the universal primers, T3 and T7, in various combinations with degenerate primers based upon published rap-1 genes of alternative Babesia spp. Amplified PCR products representing partial Bdrap-1 sequences were separated on 1% agarose gels, cleaned using QiAquick gel extraction kit (Qiagen), cloned into TOPO TA vector (Life Technologies Corporation, Carlsbad, CA), and transformed into TOP10 E. coli strain (Life Technologies Corporation, Carlsbad, CA) prior to sequencing. Partial Bdrap-1 sequences obtained were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to design specific Bdrap-1 primers. Using various combinations of the T3 and T7 universal and Bdrap-1 specific primers, the full length sequence of the cDNA Bdrap-1 gene was obtained using the same amplification, and cloning strategy as above. The genomic sequence of the Bdrap-1 gene was obtained by amplifying 100 ng genomic B. divergens DNA (prepared as described in [25]) using specific primers Bdrap-1F1 (5'-GCTTTCGAAGCTTATTGACTCT-3') and Bdrap-1R1 (5'-GTAAAAACTTGGAGTAATTTGGTGA-3'). The PCR product was separated on 1% agarose gel and cleaned using QiAquick gel extraction kit (Qiagen, Hilden, Germany), cloned into TOPO TA vector (Life Technologies Corporation, Carlsbad, CA), and transformed into TOP10 E. coli strain (Life Technologies Corporation, Carlsbad, CA) prior to sequencing.

Protein Expression and purification and rabbit antiserum production

Genomic B. divergens DNA encoding residues Val32 to Met579 of the BdRap-1 protein was amplified using the modified primers Bdrap-1F2 (5'-CCGGGATCCGTGGCTCTTGGGCTCGAG-3'), containing BamHI restriction site, and Bdrap-1R2 (5'-CGCGGATCCAGAGTCAGGTCATGGCTT-3'), containing XhoI restriction site and stop codon, using SuperMix kit (Life Technologies Corporation, Carlsbad, CA). Amplified products were cloned into the expression vector, pET28a vector (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer’s instructions. E. coli strain BL21 was transformed with pET28a-Bdrap1 in 250 mL of SOB medium containing 100 µg/mL Ampicillin (Sigma-Aldrich, St Louis, MO) and inoculated with 1 mL of fresh overnight culture and grown at 37°C to A600 = 0.6 prior to induction with 0.2 isopropyl-ß-D-thiogalactopyranoside. After 4 h of induction, recombinant BdRap-1 protein was purified from bacterial cells using Ni-NTA Buerl kit as per manufactures instructions (EMD Millipore Corporation, Billerica, MA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). The insoluble material was removed by centrifugation and the soluble fraction was used to purify the rBdRAP-1 protein. Protein concentration was determined by the Bradford’s Dye (Bio-Rad Laboratories, Hercules, CA). Polyclonal
serum was then raised against the rBdRAP-1 in rabbits (Strategic Diagnostics Inc., Newark, DE).

**Immunoprecipitation**

Freshly cultured parasites were washed and resuspended in methionine-free RPMI 1640 (MP Biomedicals Inc., Aurora, OH). 200 μCi/mL of [35S] methionine/cysteine (PerkinElmer, Boston, MA) was added and parasites were incubated at 37°C for 2 h. Parasites were lysed in NETT buffer [10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100] using a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) and centrifuged to collect the supernatant. Lysates were pre-cleared with Protein G-Sepharose beads (GE Healthcare, Waukesha, WI) before antibody addition. Protein G-Sepharose beads were added and washed extensively with NETTS (10 mM Tris, pH 7.5, 500 mM NaCl, 5 mM EDTA and 0.1% Triton X-100) and NETT buffers. Protein was eluted from the beads using elution buffer [10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA], boiled and run on SDS-PAGE gel. Gels were fixed with 25% Isopropanol alcohol; 10% Acetic Acid for 30 min and enhanced with Amplify™ fluorographic solution (GE Healthcare, Waukesha, WI) for 45 min, dried under vacuum, and exposed to X-ray film for autoradiography.

**Immunofluorescence assay (IFA)**

Cultured parasites with a high parasitaemia (~70%) or purified free merozoites were prepared for immunofluorescence (IFA). The parasites were smeared on glass slides and fixed in cold 90% acetone/10% methanol. The slides were incubated for 30 min with purified anti-rBdRAP-1 antibodies diluted 1:200 in 1× PBS/1% BSA. The slides were washed three times in 1× PBS and incubated for 30 min with a polyclonal anti-rabbit immunoglobulin (Dako, Productionsvæj Denmark) diluted 1:200 in 1× PBS/1% BSA. The slides were washed again three times in 1× PBS, rinsed once in distilled water, and mounted using Vectashield with DAPI solution for microscopy (Vector laboratories Inc., Burlingame, CA).

**Electron microscopy**

Cultured parasites and free merozoites were fixed with 1% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, for 1 h at 4°C, washed in 0.1 M buffer, pH 7.4 and treated with 50 mM ammonium chloride to quench the remaining aldehydes. The fixed infected RBCs and the free merozoites were than dehydrated and embedded in LR-White Resin (Electron Microscope Sciences, Hatfield, PA). Thin sections of embedded parasites were mounted on parlond covered nickel grids, blocked in 2% BSA and probed with purified anti-rBdRAP-1 antibodies overnight at 4°C, washed in buffer containing 1× BSA and Tween-20 and incubated with goat-anti-Rabbit IgG coupled with 6 nm gold particles (Electron Microscopy Sciences, Hatfield, PA) or Goat-anti-Mouse IgG coupled with 5 nm gold particles (GE Healthcare, Waukesha, WI). After staining with uranyl acetate, sections were observed using a Philips-410 electron microscope.

**RBC Binding assays**

**RBCs.** Erythrocytes collected in 10% citrate-phosphate-dextrose were washed 3 times in 1× PBS and treated with 1.0 mg/mL of neumaminidase and chymotrypsin, and 0.1 and 1.0 mg/mL of trypsin as described [25] or treated with Proteinase K (Life Technologies Corporation, Carlsbad, CA) and papain (Sigma-Aldrich, St Louis, MO) as follows: 500 uL (packed cell volume) of washed erythrocytes were incubated with 0.5 mg/mL Proteinase K for 30 in at 37°C in a final volume of 4 mL with 1× PBS. 500 uL (pvc) of washed erythrocytes were incubated with 0.5 mg/mL Papain at 30 min in a total volume of 2 mL.

**Parasite Supernatant.** Freshly cultured parasites were washed and re-suspended in methionine-free RPMI 1640 (MP Biomedicals Inc., Aurora, OH). 200 μCi/mL of [35S] methionine/cysteine (PerkinElmer, Boston, MA) was added, and parasites were incubated at 37°C overnight. The supernatant was collected by centrifugation at 14,000 rpm for 15 min and a protease inhibitor mixture (Sigma-Aldrich, St Louis, MO) was added. The parasite supernatant was pre-cleared with protein G (GE Healthcare, Waukesha, WI) before to use.

**Binding assay.** An aliquot of 500 uL of labelled parasite supernatant, confirmed as having BdR-1 by immunoprecipitation, was mixed with 100 uL target erythrocytes (wild type or enzyme treated) for 30 min at r.t. The mixture was then spun at 6000 g for 1 min through sodium phthalate oil to remove unbound material. Oil and supernatant was aspirated, leaving the RBC/protein complexes. Cells were lysed in NETT [0.5% Triton-X100, 150 mM NaCl, 10 mM EDTA 50 mM Tris, pH 7.4] for 30 minutes at 4°C and spun at 14, 000 rpm for 5 min. The soluble extract containing the BdR-1-receptor complex was immunoprecipitated with anti-rBdRAP1 antibodies. All experiments were repeated at least 3 times with identical pattern of binding observed.

**ELISAs**

Microtiter ELISA plates (Costar, Corning Life Sciences, Corning, NY) were coated with 1 μg/mL of recombinant BdRAP-1 protein.
diluted in 0.05 M carbonate buffer, pH 9.6. After incubation overnight at 4°C, the plates were washed 5 times with 1× phosphate-buffered saline (PBS) with 0.05% Tween-20 (1× PBS-T) and blocked with blocking buffer (3% BSA in 1× PBS-T) for 1 h at 37°C. Serum samples, diluted in blocking buffer, were reacted with the bound antigens by incubating for 1 h at 37°C, in triplicate wells. Serum from the cows was diluted from 1:200 to 1:12,800, and serum from the gerbils was diluted 1:200 to 1:25,600. The bound antibodies in the cow sera were detected after incubation for 1 h at 37°C with goat anti-cow conjugated HRP at 1:1000 (Abcam, Cambridge, MA) diluted in blocking buffer; antibodies in the gerbil sera were detected after incubation for 1 h at 37°C with goat anti-rat conjugated HRP (Pierce Antibody Products, Thermo Fisher Scientific Inc, Rockford, IL) diluted 1:10,000 in blocking buffer. Tetramethylbenzidine (Sigma, St Louis, MO) was used as the substrate for all ELISAs for 30 min. Sulphuric acid (2M) was used to stop the substrate reaction, and the optical density (OD) was read at 450 nm immediately on a SpectraMax 190 ELISA Reader (Molecular Devices, Sunnyvale, CA).

In vitro growth inhibition assays (GIA)

Total preimmune rabbit (PI) and BdRAP-1 IgGs were purified using protein-G Sepharose (GE Healthcare, Waukesha, WI) with IgG binding and elution buffers (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s recommendation and dialyzed with DPBS. Purified pre-immune IgG (Sigma-Aldrich, St Louis, MO) and rBdRAP1 were added to pre-warmed medium and 5% haematocrit of RBCs at ~4% parasitaemia. The final concentration of antibodies in the GIA was 2 mg/mL in a 500 ul final volume. Each antibody was tested in triplicate. The culture medium was removed at 24 h and replaced with fresh medium, 10% human serum, and purified IgG to a final concentration of 2 mg/mL. Samples were incubated at 37°C with 90% CO2, 5% nitrogen, 5% oxygen. Smears were made at 0 h, 12 h, 24 h, 36 h. The slides were fixed in 100% methanol and stained with Giemsa (Sigma-Aldrich, St Louis, MO). Parasitaemia was determined after counting the total number of intracellular parasites present in 1×10⁴ RBC at ×100 magnification using a Nikon Eclipse E 600 microscope, and the invasion inhibition with respect to the controls was determined for each antibody tested.

Results and Discussion

Cloning of a RAP-1 homolog (BdRAP-1) of B. divergensRouen1987

The B. divergens genome has not yet been sequenced. Although a B. divergens RAP-1 sequence from a bovine infection has been identified [19], no BdRAP-1 homolog from a human infection has been identified, and it is possible that sequence polymorphisms may exist between strains that have alternative host preferences to enable specific host cell interactions. To clone the gene encoding BdRAP-1, a B. divergens cDNA library, created from material obtained from a strain isolated from a human B. divergens infection and maintained in culture [20,22], was screened by PCR with the universal primers, T3/T7, in conjunction with degenerate primers based upon homology within published rap-1 genes of Babesia divergens RAP-1

Figure 3. Native BdRAP-1 localised to apical complex. A, Immunofluorescence staining with anti-rBdRAP-1 antibodies (FITC) on fixed cells infected with B. divergens parasites (DAPI) clearly shows that BdRAP-1 is localised to the apical ends of all 4 parasites in this Maltese Cross form of the intracellular parasite (see Merged panel). B, Electron microscopy of a singly-infected RBC (direct magnification of 30,000×, print magnification of ~50,000×) shows the location of the rhoptry organelles and the presence of BdRAP-1. C, An enlarged section (direct magnification 49,000×, print magnification of ~140,000×) clearly shows localisation of native BdRAP-1 is within the bulb of the rhoptry organelle. doi:10.1371/journal.pone.0107727.g003
Comparison of BdRAP-1 sequence with other Apicomplexan sequences

Conceptual translation of the 1,225 bp open reading frame resulted in prediction of a ~46 kDa protein. A BLAST search with the full translated sequence identifies the region encompassing Ala41 to Gly291 as a member of the RAP-1 superfamily, as shown in Figure 1. The BdRAP-1 antigen we identified shows a 93% homology to a putative B. divergens RAP-1 protein isolated from a bovine infection [19], 47% identity to B. canis [20], and 33% identity to B. bovis [27], although there is very little identity to RAP-1 antigens of either Plasmodium or Toxoplasma spp (not shown). Sequence alignment with these RAP-1 proteins from other babesial species shows the N-terminal region that encompasses the predicted location of the RAP-1 superfamily in B. divergens is relatively conserved (~40% homology) in both B. canis and B. bovis, and includes 4 conserved cysteine residues across the species, indicated by bold underline in Figure 1. There are 19 non-synonymous polymorphisms between the BdRAP-1 amino acid sequence identified here from a human infection and the BdRAP-1 identified from a bovine infection (highlighted in grey in Figure 1). Although these polymorphisms appear spread throughout the amino acid sequence, it is interesting to note that all but one of these occur downstream of the 4 conserved cysteine residues, suggesting the N-terminal region is the location of the RBC receptor binding site for all babesial RAP-1 antigens. It remains to be determined if the polymorphisms have a role in determining host cell preference for parasite invasion.

Expression of BdRAP-1 as a hexa-His fusion protein and generation of antibodies

The sequence encoding Val32 to Met779 of BdRAP-1 was cloned into the expression vector pET28(a) and expressed in E. coli as a HIS fusion protein. The predicted mass of the recombinant product was ~42 kDa, including the hexa-His tag. The recombinant BdRAP-1 protein was purified from the soluble fraction and Coomassie staining shows the purified BdRAP-1 recombinant product is observed at ~42 kDa (data not shown), as predicted from the gene sequence, and was used to immunize rabbits.

BdRAP-1 is expressed as a 46 kDa protein in the erythrocytic parasite

Immune sera from rabbits immunized with the purified rBdRAP-1 protein were used to characterize the native protein in the parasite. Immuno-blots with native parasite lysate from infected cells showed a distinct single band of 46 kDa (Figure 2A, lane 1), but no products were detected with pre-immune sera (Figure 2A, lane 2). Immunoprecipitation (IP) with lysate from infected cells detected the same single band of 46 kDa, confirming the size of the native BdRAP-1 protein (Figure 2B, lane 1). Many rhoptry proteins undergo proteolytic processing during invasion. To investigate if BdRAP-1 was similarly processed, immunoprecipitations were carried out on spent culture supernatants from in vitro cultures of the parasite. These experiments revealed the 46 kDa native BdRAP-1 (Figure 2B, lane 2) along with a secondary, lower molecular-weight band of ~20 kDa (Figure 2B, lane 2). These analyses suggest that the native BdRAP-1 undergoes proteolytic processing, similar to RAP-1 of P. falciparum [26,27].

BdRAP-1 localizes to the rhoptry organelles in the parasite

All the RAP-1 proteins that have been described so far have been localized to the apical, invasive end of the merozoite, and should be true of BdRAP-1 if it also plays a role in parasite entry. The subcellular localization of BdRAP-1 was determined by immunofluorescence assay (IFA) using anti-rBdRAP-1 rabbit antibodies. Figure 3A shows the staining pattern obtained on smears of asexual parasites demonstrating that BdRAP-1 is indeed
located at the apical end of merozoites. The punctate staining in this region, wherein a single dot occasionally resolves into double foci, is a hallmark of rhoptry localization. We confirmed this specific localization of BdRAP-1 in rhoptries by immunoelectronmicroscopic analysis (IEM). The IEM was carried out on sections of parasite-infected RBC with anti-rBdRAP-1 antibodies. As can be seen in Figure 3B, discrete antibody reactivity was observed in these electron-dense organelles with the morphological characteristics of rhoptries (indicated by arrows in Figure 3B). Increased magnification showed that BdRAP-1 is localized to the bulb of the rhoptries (Figure 3C), and thus may have an active role in host cell invasion.

BdRAP-1 binds to erythrocytes via a trypsin-,
chymotrypsin- and neuraminidase-resistant RBC receptor

Parasite proteins that mediate interaction with host receptors during invasion are commonly localized in the apical organelles. Having established its location at the invasive apical end of the merozoite and homology with other known Babesia RAP-1 proteins, we performed assays to determine whether native BdRAP-1 binds to RBCs. Members of the RAP-1 protein family in Babesia are involved during the complex process of invasion of host RBCs through direct binding with receptor(s) on the host-cell surface [1,30]. Identifying both the parasite ligands and their complementary RBC receptor in each part of the invasion process is critical to understanding the invasion biology of B. divergens in the human host, and provides continuing information for the development of effective intervention and prevention strategies. Studies have shown that extracellular merozoites release parasite proteins into the culture, and such culture supernatants can be a source of parasite ligands that bind erythrocytes. Thus, [35S]methionine/cysteine-labeled spent merozoite supernatants were used as the source of RBC-binding proteins, and when the eluate was immunoprecipitated with anti-rBdRAP-1 antiserum, a dominant band at -46 kDa was seen (Figure 4, Panel A, lane WT). Thus,
Anti-BdRAP-1 antibodies do not significantly inhibit invasion in vitro

Purified IgG from the rabbit anti-rBdRAP-1 serum was used to evaluate the role of native BdRAP-1 during the invasion process. Cultures were established with a starting parasitaemia of 11,300 parasites/mL and infected with Babesia divergens parasites. Recipients of blood products are at greater risk of developing severe babesiosis due to factors such as extremes in age, lack of a spleen, hemoglobinopathies, cancers, HIV, and use of immunosuppressive therapy [31], yet the donor population largely consists of healthy, asymptomatic adults. The high reactivity also suggests that BdRAP-1 might be a potential vaccine candidate.
in the presence of anti-BdRAP-1 antibodies after 12 h was 11% (Figure 6), and the maximum inhibition observed was 19% after 36 h. However, there was no significant difference in the growth inhibition observed for the pre-immune serum, which showed 14% and 21% inhibition at 12 h and 36 h, respectively. The limited growth inhibition observed suggests the binding of BdRAP-1 to the RBC receptor during invasion is not the primary pathway this species uses to invade host cells, or, there is significant redundancy in the invasion mechanisms and multiple invasion pathways are available to the parasite. Additionally, the full repertoire of RAP-1 and other RAP-1 related molecules in the parasite genome has not been accounted for, as it has in other Babesia species [32–35], and thus, homologs of this antigen may serve in its role when BdRAP-1 is neutralized by antibodies. It seems increasingly likely that RAP-1 in malaria may not directly play a role in the invasive process, but like other proteins housed in the rhoptry bulb, may participate in events downstream of active entry, like enhancing parasite survival in the host cell [36–38]. Thus, binding of BdRAP-1 to RBCs may not reflect an in vivo role for the ligand but relate to potential interactions that would occur between RAP-1 and RBC proteins transferred to the parasitophorous vacuole membrane (PVM) during invasion, or maybe the result of non-specific binding of the ligand to lipid membranes, including both the RBC surface and the PVM. Future experiments directed to the dissection of this interaction may help elucidate the role of these rhoty proteins.

Conclusions
Although other Babesia RAP-1 proteins have been identified [19,26,27,39,40], we present the gene encoding a RAP-1 homologue of a human babesia parasite BdRouen1987, the ~46 kDa Babesia divergens RAP-1 protein and have characterized this important antigen. BdRAP-1 is located in the bulb of the rhoptries and binds to an RBC receptor(s) that is resistant to sialidases and various proteinases. We do not find significant reduction in growth with anti-RAP1 in vitro, suggesting the presence of alternative pathways of invasion or alternative roles for this ligand. Identification and assessment of the antigens used for RBC invasion and remodelling of RBC after invasion are essential for understanding the invasion biology of B. divergens to determine the most effective interventions.

Author Contributions
Conceived and designed the experiments: MR AA RO JCS JG CAL. Performed the experiments: MR AA RO JCS MS. Analyzed the data: MR AA RO JCS MS JG CAL. Contributed reagents/materials/analysis tools: MR AA RO JCS MS JG CAL. Contributed to the writing of the manuscript: RO JG CAL.

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