**Babesia divergens** Apical Membrane Antigen 1 and Its Interaction with the Human Red Blood Cell

Estrella Montero, Marilis Rodriguez, Yelena Oksov, and Cheryl A. Lobo*

Department of Blood-Borne Parasites, Lindsley F. Kimball Research Institute, The New York Blood Center, New York, New York 10021

Received 1 August 2008/Returned for modification 8 October 2008/Accepted 25 August 2009

Multiple parasite ligand-erythrocyte receptor interactions must occur for successful *Babesia* and *Plasmodium* invasion of the human red cell. One such parasite ligand is the apical membrane antigen 1 (AMA1) which is a conserved apicomplexan protein present in the micronemes and then secreted onto the surface of the merozoite. Much evidence exists for a vital role for AMA1 in host cell invasion; however, its interaction with the host erythrocyte has remained controversial. In this paper, we present a detailed characterization of a *Babesia divergens* homolog of AMA1 (BdAMA1), and taking advantage of the relatively high amounts of native BdAMA1 available from the parasite culture system, show that proteolytic products of native BdAMA1 bind to a trypsin- and chymotrypsin-sensitive receptor on the red blood cell. Moreover, immuno-electron microscopic images of the *B. divergens* merozoite captured during invasion offer additional evidence of the presence of BdAMA1 on the red cell membrane. Given the importance of AMA1 in invasion and the central role invasion plays in pathogenesis, these studies have implications both for novel drug design and for the development of new vaccine approaches aimed at interfering with AMA1 function.

The genus *Babesia* comprises more than 100 species of protozoan pathogens that infect erythrocytes of a wide variety of vertebrate hosts (30). The organisms are transmitted by their tick vectors during the taking of a blood meal from the vertebrate host (21, 30). Babesiosis has long been recognized as an economically important disease of cattle, but only in the last 30 years has *Babesia* been recognized as an important pathogen in humans. Human babesiosis is caused by one of several babesial species that have distinct geographical distributions based on the presence of competent animal hosts (15). In Europe, babesiosis in humans is caused by the bovine pathogen *Babesia divergens* (12). In North America, human babesiosis is caused predominantly by *Babesia microti* (8), a rodent-borne parasite.

The spectrum of human babesiosis is broad, ranging from an apparently silent infection to a fulminant, malaria-like disease, resulting occasionally in death. When present, symptoms typically are nonspecific (fever, headache, and myalgia) (27). This pathology of babesiosis, like malaria, is a consequence of the parasitemia which develops through the cyclical asexual replication of *Babesia* parasites in a patient’s red blood cells (RBCs). The parasite’s ability first to recognize and then to invade RBCs is central to the disease process, and thus *Babesia* molecules involved in these recognition and invasion steps are of great interest for the development of prophylaxis. Additionally, because of the parallels in the invasion patterns of *Plasmodium* and *Babesia* into human erythrocytes, there is keen interest in developing *B. divergens* as a model to study malarial RBC invasion. Two of the major difficulties of studying *Plasmodium falciparum* invasion can be overcome in the *B. divergens* invasion assay system since high parasitemia (>80%) and infectious free merozoites are obtained in *B. divergens* in vitro cultures (34). Thus, such research could impact malaria studies as well.

Apicomplexan organisms are defined by a common set of apically located secretory organelles required for host cell invasion, which utilizes a mechanism having many conserved features. This is especially true for *P. falciparum* and *B. divergens* because they share a host cell, the human erythrocyte that they must invade, to establish their asexual cycle. Invasion is accompanied by exocytosis of the contents of various secretory organelles. In accord with their diverse morphologies, secretion from these apical organelles occurs at distinct stages of invasion. The process is not fully characterized in *Plasmodium*, but studies in *Toxoplasma* suggest that microneme secretion takes place at an early stage in invasion and initiates junction formation (7). One such molecular secretion from the micronemes is apical membrane antigen 1 (AMA1) that has been identified as a conserved antigenic protein in all *Plasmodium* species as well as *Babesia bovis*, *Plasmodium gilsoni*, and *Toxoplasma gondii* (11, 13, 35). It is essential for host cell invasion (33), but its role is still incompletely understood. *Plasmodium* AMA1 has been shown to be localized to the micronemes of developing intraacellular parasites (5) and to the apical surface of extracellular parasites just prior to invasion (25). In an elegant series of electron microscopic images, Mitchell and others showed that in the presence of anti-AMA1 antibody, the *Plasmodium knowlesi* merozoite failed to undergo junction formation and hypothesized that AMA1 plays an important role in apical reorientation (23). It is a prime candidate for inclusion in a malaria vaccine as vaccination with recombinant *Plasmodium* AMA1 has been demonstrated to induce protective immunity against a homologous parasite challenge in many malarial models (6, 28, 31).

In this study, we report the cloning and characterization of an AMA1 homolog of *B. divergens* (BdAMA1) and provide
novel insights into parasite invasion by a detailed study of the molecular interactions of BdAMA1 with human erythrocytes.

**MATERIALS AND METHODS**

**Parasite propagation.** Blood stage cultures of *B. divergens* were maintained in vitro in human A⁺ blood using RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% human serum and 7.5% (w/v) sodium bicarbonate solution (Invitrogen Corp.). Cells were cultured at 37°C in 90% CO₂, 5% nitrogen, and 5% oxygen.

**gDNA and total RNA isolation.** Genomic DNA (gDNA) and total RNA were isolated from *B. divergens* cultures with ~60% of parasitemia. gDNA was prepared using a pellet of infected RBCs. The parasite pellet was incubated in DNA lysis buffer (0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, sodium dodecyl sulfate [SDS; 0.5% by volume], and 100 μg ml⁻¹ of proteinase K) for 16 h at 50°C. Nucleic acid was recovered by phenol-chloroform extraction, followed by ethanol precipitation. RNA was removed by RNase digestion (Invitrogen Corp.), and DNA was subjected to a further round of phenol-chloroform extraction and ethanol precipitation. Total RNA was prepared using Trizol LS Reagent (Invitrogen Corp.) and chloroform extraction; DNA was removed by DNase (Invitrogen) and ethanol precipitation.

**Cloning of BdAMA1.** Degenerate oligonucleotide primers for amplification by reverse transcription-PCR (RT-PCR) were BdAMA-1F1 (5'-KRYKY AGKAGTCGGT-3') and BdAMA-1R1 (5'-TWCCCATCTCATTC-3'). The 802-bp BdAMA1 product was obtained by RT-PCR using 1 μg of total RNA. RT was performed using an RT system from Promega (Madison, WI). Then, PCR amplification of the partial coding region was done using a SuperMix kit from Invitrogen. PCR was carried out under the following conditions: 95°C for 3 min; 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. In order to complete the BdAMA1 cDNA, a *B. divergens* cDNA library (24) was screened with the AM1 cDNA PCR product target with digoxigenin (Roche, Applied Science, Indianapolis, IN), and standard protocols were followed. The positive clones were purified by the same digoxigenin probe procedure and amplified by PCR using T3 and T7 universal primers; the products were sequenced.

**PCR, PCR and RT-PCR of a genomic sequence of the BdAMA1 gene and the BdAMA1 open reading frame (ORF) were carried out using gDNA and total RNA, respectively, and the primers BdAMA-1F2 (5'-ACCTAAAAAGGGGC TAACAACAC-3') and BdAMA-1R2 (5'-GCACCTTATAGATTGGTACCG-3').** The PCR was carried out using a SuperMix kit from Invitrogen. For the genomic PCR 100 ng of *B. divergens* gDNA was used, and 1 μg of total RNA was used for the RT-PCR. Most of the amplified products were subcloned into TOPO TA vector (Invitrogen Corp.) for sequencing. The constructions were maintained in the Escherichia coli TOP10 strain (Invitrogen) and then sequenced on both strands. All sequencing reactions were performed by the dye-terminator (Sequenase) method using custom synthesized primers. DNA sequences and predicted amino acid sequence comparisons were performed with GenBank, EMBL, DDBJ, and PDB and all nonredundant GenBank coding sequence translations in the PDB, SwissProt, PfIR, and PRF databases using BLAST and the Clustal W(http://align.genome.jp/).

**Protein expression and purification and rabbit antiserum production.** PCR and RT-PCR of a genomic sequence of the BdAMA1 gene and the BdAMA1 open reading frame (ORF) were carried out using gDNA and total RNA, respectively, and the primers BdAMA-1F3 (5'-ACCTAAAAAGGGGC TAACAACAC-3') and BdAMA-1R3 (5'-GAAGTCGGTATGTCGGGAC-3'). The 802-bp BdAMA1 product was obtained by RT-PCR using 1 μg of total RNA. RT was performed using an RT system from Promega (Madison, WI). Then, PCR amplification of the partial coding region was done using a SuperMix kit from Invitrogen. PCR was carried out under the following conditions: 95°C for 3 min; 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. In order to complete the BdAMA1 cDNA, a *B. divergens* cDNA library (24) was screened with the AM1 cDNA PCR product target with digoxigenin (Roche, Applied Science, Indianapolis, IN), and standard protocols were followed. The positive clones were purified by the same digoxigenin probe procedure and amplified by PCR using T3 and T7 universal primers; the products were sequenced.

**Hydrophobic epitopes.** The three-dimensional structure of transmembrane helices in proteins (Center for Biological Sequence Analysis, National Institutes of Health (3)). Nucleotide sequence alignments were made by BLAST and the Clustal W(http://align.genome.jp/).

**Isolation of free merozoites.** Freshly cultured parasites were washed and resuspended in methionine-free medium (RPMI 1640 medium; MP Biomedicals, Inc., Solon, OH). A 3% (w/v) solution of methionine-sulfone (Merck, Elmer, MA, Boston) 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) and centrifuged to collect the supernatant. Lysates were preclarified with protein G (Amersham Biosciences) before antibody addition. Protein-G Sepharose beads were added and washed extensively with NETT buffer (10 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100) and NETT buffer. Protein-G Sepharose beads were eluted from the beads using 10 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM EDTA buffer; boiled; and run on SDS-polyacrylamide gel electrophoresis gels. Gels were stained with Coomassie blue R-250, fixed with fixing solution (25% isopropanol alcohol and 10% acetic acid) for 30 min, enhanced with Amplify fluorographic solution (Amersham Biosciences) for 45 min, dried under vacuum, and exposed to film for autoradiography.

**Immunoprecipitation.** Freshly cultured parasites were washed and resuspended in methionine-free medium (RPMI 1640 medium; MP Biomedicals, Inc., Solon, OH). A 3% (w/v) solution of methionine-sulfone (Merck, Elmer, MA, Boston) 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) and centrifuged to collect the supernatant. Lysates were preclarified with protein G (Amersham Biosciences) before antibody addition. Protein-G Sepharose beads were added and washed extensively with NETT buffer (10 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100) and NETT buffer. Protein-G Sepharose beads were eluted from the beads using 10 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM EDTA buffer; boiled; and run on SDS-polyacrylamide gel electrophoresis gels. Gels were stained with Coomassie blue R-250, fixed with fixing solution (25% isopropanol alcohol and 10% acetic acid) for 30 min, enhanced with Amplify fluorographic solution (Amersham Biosciences) for 45 min, dried under vacuum, and exposed to film for autoradiography.

**Isolation of free merozoites.** Free, viable merozoites were obtained using cultures of ~60 to 80% parasitemia following a protocol described in Valentin et al. (34). Forty milliliters of supernatant yielded ~10⁶ merozoites. Free merozoites were smeared onto slides for immunofluorescence assays (IFA) and fixed with 1% paraformaldehyde for electron microscopy or resuspended in RPMI 1640 medium for use in vitro invasion assays.

**In vitro invasion assays and electron microscopy studies.** Invasion assays were performed using purified free merozoites. Cultures were prepared by warming RPMI medium supplemented with 10% of human serum and 5% hemocrit of RBCCs (6 × 10⁶) at 37°C in 90% CO₂, 5% nitrogen, and 5% oxygen for 30 min. Then, 2 × 10⁶ free merozoites were added to the warmed medium in triplicate. Samples were incubated at 37°C with 90% CO₂, 5% nitrogen, and 5% oxygen, and samples were fixed with 1% paraformaldehyde for electron microscopy fixed at 1, 3, 5, and 10 min.

**IFA.** Cultured parasites with a high parasitemia (70%) or purified free merozoites were prepared for indirect surface IFA. The parasites were smeared on glass slides and fixed in cold acetone. The slides were incubated for 30 min with BdAMA1-e antibodies diluted 1:40 in phosphate-buffered saline (PBS). The slides were washed three times in PBS and incubated for 30 min with a polyclonal anti-rabbit Ig (Dako, Produktionsvej, Denmark) diluted 1:200 in PBS. After the last three washes in PBS, the slides were rinsed in distilled water and mounted using Vectorshield with DAPI solution for microscopy (Vector laboratories, Inc., Burlingame, CA).

**Electron microscopy.** Cultured parasites and free *B. divergens* merozoites were fixed with 1% paraformaldehyde and 0.1% glutaraldehyde in 1 M cacodylate buffer for 1 h at 4°C, washed in 0.1 M buffer (pH 7.4), and treated with 30 mM ammonium chloride to quench the remaining aldehydes. The fixed infected RBCs and the free merozoites were then dehydrated and embedded in LR White resin (Electron Microscope Sciences, Hatfield, PA). Thin sections of embedded parasites were mounted on parlodion-covered nickel grids, blocked in 2% bovine serum albumin, probed with BdAMA1-e antibodies (affinity purified on rBdAMA1-e overnight at 4°C, washed in buffer containing bovine serum albumin with 0.1% Swabster’s buffer, pH 7.4, and processed as described by the manufacturer, standard, and exposed to films. The slides were stained with uranyl acetate and lead citrate and viewed with a Hitachi H-7650 transmission electron microscope at an operating voltage of 80 kV.
and Tween 20, and incubated with goat anti-rabbit IgG coupled with 6-nm gold particles (Electron Microscopy Science) or goat anti-mouse IgG coupled with 5-nm gold particles (Amersham Biosciences). After sections were stained with uranyl acetate, they were observed using a Philips 410 electron microscope (Holland).

**RBC binding assays.** (i) RBCs. Erythrocytes collected in 10% citrate-phosphate-dextrose were washed three times in PBS and treated with the various enzymes as described previously (22). Briefly, 0.2 ml of packed RBCs was treated with either 2 ml of a 1 mg/ml concentration of trypsin (treated with tosylsulfonyl phenylalanyl chloromethyl ketone; Sigma), 1 ml of a 1 mg/ml concentration of chymotrypsin (Sigma), or 0.1 ml of a 0.1 IU/ml concentration of neuraminidase (Sigma) for 30 min at 37°C. Cells treated with trypsin were then washed and treated with 0.5 mg/ml soybean trypsin inhibitor (Sigma) for 15 min at room temperature, as described earlier (22). The efficacy of each enzyme treatment was assessed in the Laboratory of Immunohematology, New York Blood Center, by assaying for the loss of RBC agglutinability using a panel of monoclonal antibodies against suitable antigenic determinants on different blood group proteins.

(ii) Parasite supernatant. Freshly cultured parasites (3.5 × 10^7) were washed and resuspended in 10 ml of methionine-free medium (RPMI 1640 medium; MP Biomedicals, Inc., Aurora, OH). A total of 200 μCi ml⁻¹ of [³⁵S]methionine-cysteine (Perkin Elmer Life Sciences, 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 protease inhibitor mixture (Sigma Aldrich) was added. The parasite supernatant was precleared with protein G (Amersham Bioscience, Piscataway, NJ) before use.

(iii) Binding assay. Aliquots of 500 μl of labeled parasite supernatant were mixed with 1 ml of 1 × 10⁹ erythrocytes (with the enzyme treated) for 30 min at room temperature. The mixture was then spun at 6,000 × g for 1 min through sodium phthalate to remove unbound material. RBCs pelleted at the bottom of the tube, along with the parasites proteins bound to them, were recovered by puncturing the tube. Cells were lysed in NETT buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.4) for 30 min at 4°C and spun at 14,000 rpm for 5 min. The soluble extract containing the BdAMA1 receptor complex was immunoprecipitated with anti-BdAMA1 antibodies. All experiments were repeated at least three times with identical patterns of binding. To assess specificity of binding, 50 pg of labeled cultured supernatant was run along with the RBC binding parasite proteins, which were eluted using 1.5 M NaCl in RPMI medium. As a negative control to ensure lack of nonspecific binding, B. divergens Sub1 (BdSub1) was tested for binding, using antibodies described earlier (25).

**RESULTS**

**Cloning of BdAMA1 by RT-PCR.** To clone the gene encoding BdAMA1, an RT-PCR was carried out using degenerate primers, based on B. bovis AMA1 sequence (11) and total B. divergens RNA. A band of ~800 bp was obtained and sequenced in both directions. BLAST analysis of the nucleotide sequence showed high homology to AMA1 from different species (see below). To obtain the complete Bdama1 cDNA, a B. divergens cDNA library was screened with the Bdama1 PCR product labeled with digoxigenin. A fragment of around 2,200 bp was amplified, subcloned into TOPO TA vector, and sequenced. The cDNA clone showed a single contiguous sequence 2,122 bp long containing an uninterrupted ORF of 1,823 bp. The initial ATG showed a purine in the +3 position upstream and a guanine in the +4 position downstream (20) as well as seven stop codons before the initial ATG in the 5’ untranslated region protein as well as the nucleotide sequence of the neighboring upstream (239 bp) noncoding region, shown in Fig. 1A. The 3’ untranslated region of 62 bp finished in a poly(A) tail.

**Bdama1 gene encodes BdAMA1.** PCR amplification of the Bdama1 gene from gDNA with primers BdAMA-1F2 and BdAMA-1R2, derived from both the 5’ and 3’ ends of the Bdama1 cDNA, produced a single DNA fragment of 1,823 bp. The ORF encoded a protein (BdAMA1) of 606 amino acids with an estimated molecular mass of 67,598 Da and an isoelectric point of 5.84. Amplification from total RNA under the same conditions produced a fragment of the same size. The coding regions of the cDNA and Bdama1 gene were cloned into the TOPO TA vector and sequenced. The nucleotide sequences of both products were identical, suggesting that the Bdama1 gene contains no introns. Figure 1A shows a cartoon of the gene highlighting the features of the Bdama1 gene. A BLAST search of the nondegenerate protein databases with the deduced BdAMA1 protein sequence showed that it possesses significant similarity to other known apical membrane antigens, especially that of B. bovis with 51% identity (score of 638) and P. falciparum with 30% identity (score of 217), and a lower score of 64 with the AMA of the related apicomplexan T. gondii. BdAMA1 is thought to be initially synthesized with a putative signal peptide 31 residues long, with the most likely cleavage site between positions Arg⁵⁰ and Glu⁶¹. The signal peptide probability, predicted by SignalP, version 3.0, was 0.811. After cleavage of the signal peptide, the calculated molecular mass of the mature protein is 64.2 kDa. Moreover, the TMHMM program predicted a hydrophobic membrane region of 17 amino acids (aa) identified between Tyr²² and Thr²⁴. This region divides BdAMA1 into an amino terminal-domain of 492 aa or an N-terminal ectoplasmic region, followed by a single transmembrane region and a small or C-terminal cytoplasmic domain of 65 aa, features characteristic of the classical type I membrane proteins (Fig. 1A). Thus, the bioinformatics programs suggest that the ectodomain of BdAMA1 could start at Val⁵² and extend to the C-terminal residue Lys⁸⁵, having a predicted molecular mass of 54.5 kDa, and the cytoplasmic region likely extends from Tyr⁸⁴ to Glu⁶⁰ with an estimated mass of 7.7 kDa.

**Bdama1 gene is a single-copy gene.** It is important to establish the copy number of genes whose products are believed to be involved in important functions like invasion. Thus, to analyze the copy number of the Bdama1 gene, Southern blot analysis of the B. divergens Rouen 1986 strain was performed (Fig. 1B). B. divergens gDNA was digested with various restriction enzymes and probed under high stringency conditions with a partial Bdama1 probe of 802 bp. The hybridization patterns obtained indicated that Bdama1 is a single-copy gene. The Bdama1 cDNA contains no Sall site (Fig. 1C), and the Southern blot data therefore indicated that the whole
FIG. 1. (A) Graphic depiction of Bdana1 cDNA (GenBank accession number EU486539). The Bdana1 cDNA contains the complete ORF (1,821 bp). Noncoding regions of the gene are shown in black, and coding regions are indicated by the shaded box. The graphic representation of the predicted full-length BdAMA1 protein is also shown in the bar below. BdAMA1 is a type I integral membrane protein with characteristic structures: a signal peptide (black), an N-terminal domain (Nt) or a cysteine-rich ectodomain, a single transmembrane domain (gray), and a C-terminal (Ct) cytoplasmic tail (hatched box). Domains I, II, and III and PAN domains between the cysteine residues are defined by analogy to those described for P. knowlesi AMA1 (14). (B) Southern blot analysis. Genomic B. divergens DNA was analyzed by high stringency using the original BdAMA1 cDNA clone of 802 bp obtained by RT-PCR as a probe. B. divergens gDNA was digested with a variety of restriction enzymes: HindIII and NsII that do not digest within the gene and BamHI, EcoRI, KpnI, SalI, and XhoI that digest within the gene. Ten micrograms of each digest was electrophoresed on a 1% agarose gel and transferred to nylon membrane. Lane 1, BamHI; lane 2, HindIII; lane 3, KpnI; lane 4, NsII; lane 5, EcoRI; lane 6, XhoI; lane 7, SalI. The migration of size markers is indicated in kilobases. (C) Restriction enzyme map of the BdAMA1 gene and the position of the cDNA probe used in the Southern blot analysis. The graph represents a single gDNA fragment of 1,823 bp (gray box) obtained by PCR using the primers derived from the both 5’ and 3’ ends of the Bdana1 cDNA. The Bdana-1F2 is in the −7 position upstream, and Bdana-1R2 is in the position +2055 downstream relative to the initial methionine of Bdana1 cDNA.

locus could be isolated on a single genomic SalI fragment of more than 9 kb (Fig. 1B).

Comparison of BdAMA1 with other apicomplexan AMA1 proteins. In all characterized Plasmodium AMA1 genes, the extracellular domain is divided into subdomains I, II, and III and stabilized by eight intradomain disulfide bridges between 16 cysteine residues (4, 14, 26). Moreover, domains I and II are structurally similar to each other and belong to the PAN module superfamily, where PAN domains are stabilized also by intradomain disulfide bridges. Notably, PAN domains are typically found in the proteins with diverse adhesion functions, binding to protein or carbohydrate receptors (32). Using the Swiss Model protein program and exploiting the crystal structures of Plasmodium vivax AMA1 (PvAMA1) (26) and P. falciparum AMA1 (PfAMA1) (4) as models, we have developed a theoretical three-dimensional structure of BdAMA1 with similar characteristics to its homologs in P. vivax and P. falciparum. The alignment of PvAMA1, PfAMA1, and BdAMA1 (Fig. 2) shows a total of 13 cysteine residues found at the same positions in the three homologs. Moreover, as can be seen from the multiple alignment (Fig. 2) and the three-dimensional structure (Fig. 3), the PAN secondary structure elements and the PAN disulfide bridge pattern are well conserved in the three parasites. Thus, the conservation of BdAMA1 residues that limited the PAN domains were observed from Cys216 to Cys236 in domain I and from Cys336 to Cys414 in domain II.

Recombinant expression of the BdAMA1 partial ectodomain in E. coli and production of polyclonal antibodies. The sequence encoding Cys138 to Ala404 that includes a region of the domains I and II from the ectodomain of BdAMA1 (Bdana-1-e) was cloned into the expression vector pGEX-6T1 and expressed in E. coli as a glutathione S-transferase (GST) fusion protein. The predicted mass of the recombinant product (GST-Bdana1-e) was ~47 kDa (data not shown). The protein was purified by affinity chromatography on a column of glutathione-agarose. The purified GST-Bdana1-e product was analyzed by Western blotting using an anti-GST monoclonal antibody and used to immunize rabbits.

Bdana1 can be identified in parasite lysates. Using a rabbit polyclonal antiserum against the rBdana1 protein produced above, three distinct AMA1 products were detected in a Western blot assay on B. divergens parasite lysate. Specifically, dominant bands at ~75 kDa (p75), ~48 kDa (p48), and ~44 kDa (p44) were identified (Fig. 4A). The Bdana1 gene predicts a protein corresponding to 68 kDa. Thus, p75 appears to represent full-length BdAMA1, and the smaller products may be processed forms of BdAMA1. Interestingly, the sizes of these bands correspond approximately to the size of the multiple forms of PfAMA1 (83 kDa, 66 kDa, 48 kDa, and 44 kDa) (17). It is known that the AMA1 in Plasmodium is originally synthesized as a high-molecular-weight molecule that requires one or more proteolytic cleavages of the precursor (9, 16). We ob-
tained identical results to those shown in Fig. 4A using anti-PfAMA1 antibody (17) on B. divergens lysates, pointing to extensive homology between the two AMA1 sequences (results not shown). These results suggested the presence of a BdAMA1 homolog similar in size and sequence to PfAMA1.

Identification of BdAMA1 coassociating proteins. Using an immunoprecipitation assay with radiolabeled B. divergens parasite lysate and anti-BdAMA1 antibody (Fig. 4B), the same three BdAMA1 bands, p75, p48, and p44, were detected. In addition, a higher-molecular-weight protein at ~145 kDa (p145) was consistently observed. Since this band was not seen in Western blots and detected only in precipitation analyses, we reasoned that p145 may be a protein that complexes with BdAMA1 and is thus pulled down in the immunoprecipitation assay. In related apicomplexans, T. gondii and P. falciparum, AMA1 has been found as a complex with rhoptry proteins (110 to 145 kDa) of the RON (for rhoptry neck) protein family (1, 2). Furthermore, this complex migrated at the moving junction during Toxoplasma invasion. Thus, p145 may represent a B. divergens homolog of one of the RON family proteins although was not consisently observed. Since this band was not seen in Western blots and detected in precipitation analyses, we reasoned that p145 may be a protein that complexes with BdAMA1 and is thus pulled down in the immunoprecipitation assay. In related apicomplexans, T. gondii and P. falciparum, AMA1 has been found as a complex with rhoptry proteins (110 to 145 kDa) of the RON (for rhoptry neck) protein family (1, 2). Furthermore, this complex migrated at the moving junction during Toxoplasma invasion. Thus, p145 may represent a B. divergens homolog of one of the RON family proteins although
this is speculation at this point. Further work will be needed to prove such an association.

BdAMA1 is secreted as a lower-molecular-weight form. To determine if BdAMA1 underwent significant proteolytic processing, a Western blot analysis was performed on concentrated, spent parasite culture supernatant. A relatively large amount of p48 was found in the extract. Two less abundant bands, p44 and p37, were also seen (Fig. 4C). However, there was no evidence of full-length BdAMA1 seen in the culture supernatant. Thus, it appears that like PfAMA1, BdAMA1 also undergoes proteolysis to release these peptides (p48, p44, and p37) into the medium.

BdAMA1 is localized at the apical end of intracellular parasites but relocates to the surface of free B. divergens merozoites. To localize BdAMA1 within the parasite, immunofluorescence assays were performed on high-parasitemia cultures, as well as on purified, extracellular, free merozoites, using the anti-BdAMA1-e antibodies. Staining of parasite DNA with DAPI solution (Fig. 5A and B) also permitted the identification of the fluorescent nuclei of the parasites. As can be seen in Fig. 5A, the staining pattern in intracellular parasites showed a concentrated fluorescence signal of the apical area (Fig. 5A, frames 3 and 4). When IFA was performed on purified merozoites, an additional fluorescence signal around the surface of the parasite was observed in addition to the apical fluorescence, (Fig. 5B, frames 3 and 4). In P. falciparum, PfAMA1 is shown to be translocated to the parasite surface from the micronemes (5). Our IFA studies point to a similar mechanism for BdAMA1. Electron microscopy using the same antibodies on sections of free merozoites confirmed the surface localization of BdAMA1 (Fig. 6A).

Temporal sequence of merozoite invasion. We also took advantage of the high viability and infectivity of purified free B. divergens merozoites and set up an invasion assay with human RBCs and tracked the invasive process using electron microscopy. This experiment had a dual purpose: (i) to formulate a series of electron microscopy images capturing the invasion of the B. divergens parasite in different steps of the process, just as was done in P. knowlesi in monkey red cells (23); and (ii) to localize the presence of BdAMA1 during the invasion process. Figure 6B shows the series of images.
from this study. The first contact between parasite and the RBC was within the first minute (minute 1) of incubation of merozoites with the RBCs (Fig. 6B, frame 1), followed by junction formation (minute 1 to 2) (Fig. 6B, frame 2), leading to invagination of the RBC membrane and deeper engulfment of the merozoite into the RBC (minute 2 to 3) (Fig. 6, frames 2 and 3). At 5 min after the assay was set up (minute 5), all merozoites were well within the RBC and could be detected as intracellular rings (Fig. 6B, frame 4). To obtain a better idea of the role of BdAMA1 during this process, we performed immuno-electron microscopy on some of the sections obtained at different time points using anti-BdAMA1-e antibodies. Free merozoites showed a clear localization of BdAMA1 on the surface of the parasites (Fig. 6A, frames 1, 2, and 3) and at around 2 to 3 min, when junctional contact was begun to be established between the merozoite and the RBC (Fig. 6C, frame 1); BdAMA1 could be seen at the moving junction of the parasite and RBC surface (Fig. 6C, frame 2).

Native BdAMA1 binds to a trypsin- and chymotrypsin-sensitive receptor on human RBCs. The apical localization of BdAMA1, its redistribution to the parasite surface, and its apparent association with the moving junction of the invading parasite, where it gets deposited on the surface of the RBC, suggested a critical role for BdAMA1 in invasion. We therefore set up an RBC binding assay using [35S]methionine-cysteine-labeled Babesia culture supernatant with RBCs, a traditional functional assay that has been used very successfully in malaria. Figure 7A shows the results of this binding assay, where it can be clearly seen that the processed products of BdAMA1, which are found in the culture supernatant, predominantly at 48 and 44 kDa, and a faint minor band of 37 kDa bind to an RBC receptor that has a trypsin- and chymotrypsin-sensitive profile but is resistant to neuraminidase (Fig. 7A). To confirm the specificity of this binding, an aliquot of the total radiolabeled culture supernatant that was used for the binding (Fig. 7B, lane 1) was run along with the total ligands eluted after binding to the RBC (Fig. 7B, lane 2). As can be seen, only a fraction of the proteins (~12 to 15 bands) found in the labeled culture supernatant actually bind the RBC. An additional control was performed by assaying for a control antigen BdSub1 in the binding eluate. As expected, although BdSub1 is present in the total culture supernatant (Fig. 7C, lane 1), it does not bind the RBC (Fig. 7C, lane 2).

Anti-BdAMA1 antibodies dramatically inhibit RBC invasion. Purified IgG from the rabbit anti-AMA1 serum was used to evaluate the importance of BdAMA1 for invasion in an inhibition of invasion assay. Purified free merozoites were used in this assay in order to discriminate effects on invasion from effects on intracellular growth. Giemsa-stained thin blood smears were prepared, and the parasitic growth was monitored after 8 h (one life cycle). A potent inhibition of parasite invasion was observed in these cultures in the presence of anti-BdAMA1-e-purified antibodies (Fig. 8). The percentage of inhibition of invasion of merozoites at 8 h postinvasion in the BdAMA1 antibody group was significantly higher than that in the control (without antibodies) or in the IgG-purified antibodies from preimmune samples (50% inhibition, with a statistically significant P value of <0.05). These results strengthen our previous findings for an erythrocyte binding role for BdAMA1, and we speculate that the anti-BdAMA1 antibody may block the interaction of AMA1 with its specific red cell receptor, thus hindering invasion.

**DISCUSSION**

Little is known about the molecules used by Babesia to attach and invade erythrocytes, and yet this is one of the most critical factors in the life cycle of the parasite. The blood stage merozoite is designed for one major role: to locate, bind to, and invade host RBCs. This is a very specific interaction; Babesia does not invade other host cells (18, 30). This specificity implies the presence of receptor(s) on the erythrocyte, which is recognized by a complementary parasite ligand(s). In this paper, we have described the cloning and characterization of one such parasite adhesin, BdAMA1, a Babesia homologue of the Plasmodium, Toxoplasma, and B. bovis AMA1 proteins. BdAMA1 is a type I membrane protein, like its other apicomplexan homologs, with an ectodomain followed by a single transmembrane region and a cytoplasmic domain. Analysis of all AMA1
FIG. 6. Electron microscopy localization of BdAMA1 on the surface of free merozoites and a general electron microscopy survey of normal invasion by *B. divergens* after addition of merozoites to fresh RBCs and immunolocalization of BdAMA1 at the junction. (A) Specific localization of BdAMA1 on the surface of the free merozoites (frames 1 and 2) by immuno-electron micrographic analysis using purified BdAMA1-e antibodies. Thin sections of resin-embedded free merozoites were probed with anti-BdAMA1 antibodies, and then bound antibodies were detected using a gold-labeled anti-rabbit IgG antibody. Bar, 250 nm. N, nucleus. Arrows show the gold particles on the surface of free merozoites. (B) Invasion was assessed after 0, 1, 3, and 5 min. At 0 min, random contacts were observed between RBCs and merozoites. (1) Apical contact is established at approximately 1 min followed by junction formation (2) and invagination of RBC membrane (1 to 2 min) and then by deeper engulfment of the merozoite (3) into the RBC (2 to 3 min). (4) Almost all parasites are well within erythrocytes at the 5-min window. Bar, 250 nm. (5) Section showing junction formation, used to parallel the immuno-EM image seen in frame 6, where the same time window section is stained with anti BdAMA1-e. Bound antibodies were detected using a gold-labeled anti-rabbit IgG antibody. BdAMA1 (arrows) can be seen deposited around the RBC membrane at the time of junction formation. (7) A section of the membranes in contact with each other demarcated in frame 6 by the rectangle is magnified to show BdAMA1 at the tight junction. Bar, 500 nm. N, nucleus.
sequences including BdAMA1 has indicated that the conserved cysteines divide the ectodomain into three distinct subdomains (4, 26). Domains I and II belong to the PAN module superfamily, suggesting that they may function in adhesion to protein or carbohydrate receptors (26).

The precise role played by AMA1 in Plasmodium invasion remains unknown. Attempts to explore its erythrocyte-binding capabilities have provided contradictory results, with the identification of either domains I and II of Plasmodium yoelii AMA1 (10) or domain III of PfAMA1 (19) being critical for this function. We took advantage of the high yields of native BdAMA1 available due to the extremely high parasitemia obtained in vitro and showed conclusively that the processed fragments of BdAMA1 (48, 44, and 37 kDa) (Fig. 7) bind RBCs to receptors that are neuraminidase resistant but chymotrypsin sensitive. Previous work with erythrocyte interactions with host cell receptors (1, 2). If the intrinsic binding affinity of AMA1 is low, as it appears to be in Plasmodium, it may be difficult to detect red cell binding in vitro. As AMA1 has such a conserved structure in apicomplexans, it was no surprise to find that, as in Plasmodium and Toxoplasma, BdAMA1 apparently also binds to high-molec-
ular-weight partners, specifically a p145 molecule. Alexander et al. (1, 2) identified these AMA1-interacting partners as molecules found in the rhoptry neck (RON) and called them RON family members. This complex was found to be a major component of the moving junction in *Toxoplasma* (2) and thus supports a direct role for AMA1 in host cell invasion. Our observations that AMA1 is present at the invasion junction and that AMA1 antibody specifically pulls down a 145-kDa molecule suggest that similar multivalent interactions may be necessary for successful *B. divergens* merozoite invasion. Further analysis of these BdAMA1 co-precipitating molecules will confirm this conservation of molecular invasion machinery.

In conclusion, we have cloned and characterized BdAMA1 and significantly extended the knowledge of its role during parasite invasion. BdAMA1 is synthesized as a ~75-kDa precursor (Fig. 4B) and is expressed at the apical end of the parasite. After parasite release from the RBC, BdAMA1 is translocated to the merozoite surface (Fig. 5), where it can be detected by both IFA and electron microscopy analysis, and is further cleaved to form ~48- and ~44-kDa proteins. These smaller fragments of BdAMA1 are found in the culture supernatant (Fig. 4C) and bind RBCs. Thus, as anticipated from the role of BdAMA1 in invasion, anti-BdAMA1 antibodies were found to have a potent inhibitory effect on parasite invasion, decreasing invasion by ~50%. Given the importance of AMA1 in all apicomplexan invasion and the central role invasion plays in pathogenesis, these studies will likely have implications for both novel chemo- and immuno-therapeutic approaches to interfering with AMA1 function.

ACKNOWLEDGMENTS

We thank Michael Blackman and Carole Long for the rabbit anti-PIfAMA1 antibodies and Luis Miguel Gonzalez and Estanislao Nistal for help with the molecular characterization of BdAMA1. This work was supported by the VP fund for new initiatives (Mohandar Narla), New York Blood Center, and the National Blood Foundation.

REFERENCES


Editor: J. F. Urban, Jr.