A Conserved Subtilisin Protease Identified in Babesia divergens Merozoites*

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Invasion of erythrocytes is an integral part of the Babesia divergens life cycle. Serine proteases have been shown to play an important role in invasion by related Apicomplexan parasites such as the malaria parasite Plasmodium falciparum. Here we demonstrate the presence of two dominant serine proteases in asexual B. divergens using a biotinylated fluorophosphonate probe. One of these active serine proteases (p48) and its precursors were recognized by anti-PfSUB1 antibodies. These antibodies were used to clone the gene encoding a serine protease using a B. divergens cDNA library. BdSUB-1 is a single copy gene with no introns. The deduced gene product (BdSUB-1) clearly belongs to the subtilisin superfamily and shows significant homology to Plasmodium subtilisins, with the highest degree of sequence identity around the four catalytic residues. Like subtilisin proteases in other Apicomplexan parasites, BdSUB-1 undergoes two steps of processing during activation in the secretory pathway being finally converted to an active form (p48). The mature protease is concentrated in merozoite dense granules, apical secretory organelles involved in erythrocyte invasion. Anti-PfSUB1 antibodies have a potent inhibitory effect on erythrocyte invasion by B. divergens merozoites in vitro. This report demonstrates conservation of the molecular machinery involved in erythrocyte invasion by these two Apicomplexan parasites and paves the way for a comparative analysis of other molecules that participate in this process in the two parasites.

Babesiosis, caused by infection with intraerythrocytic parasites of the genus Babesia, is one of the most common infections of free living animals worldwide and is gaining increasing interest as an emerging zoonosis (a disease communicable from animals to humans) (1). Babesia are transmitted by their tick vectors during the taking of a blood meal from the vertebrate host (1, 2). 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 man. Human babesiosis is caused by one of several babesial species that have distinct geographical distributions based on the presence of competent hosts (3). In North America, babesiosis is caused predominantly by Babesia microti (4), a rodent borne parasite, and also occasionally by two newly recognized species, WA1 (5) and MO-1 (6). In Europe, human babesiosis is considerably rarer but more lethal, and is caused by the bovine pathogen Babesia divergens (7). The spectrum of disease is broad, ranging from an apparently silent infection to a fulminant, malaria-like disease, which can be fatal. When present, symptoms typically are nonspecific (fever, headache, and myalgia). A number of factors have contributed to the “emergence” of human babesiosis, including increased awareness among physicians, changing ecology, and an increased population of immunocompromised individuals susceptible to infection. Because 1980, over 500 cases of human infections have been reported (9).

Parasites that live in red cells (RBCs)2 have rather ingenious ways of gaining entry to these cells. The best studied is Plasmodium spp, the etiological agent of malaria. Like Plasmodium, Babesia merozoites enter RBCs using an active invasion process that is mediated by multiple receptor-ligand interactions. The various steps in the invasion process in both Plasmodium (10, 11) and Babesia (12, 13) have been illustrated using light and electron microscopy and microcinematography. They are identical in both apicomplexans, except for the fact that soon after entry of the Babesia merozoite, the parasitophorous vacuolar membrane disappears. The invasion, growth and maturation of both Plasmodium and Babesia within the human erythrocyte is accompanied by both morphological and biochemical changes in the RBC plasma membrane, which can be attributed to the activity of specific, parasite derived factors. Parasite derived proteases are of particular importance as they play a pivotal role in both the entry and the exit of the parasite by processing both parasite adhesins and host erythrocyte proteins (14–16). Serine protease inhibitors can block invasion by

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2 The abbreviations used are: RBC, red blood cell; WA-1, B. divergens Washington-1; MO-1, B. divergens Missouri-1; BdSUB-1, B. divergens subtilisin 1; PfSUB-1, P. falciparum subtilisin 1; PfSUB-2, P. falciparum subtilisin 2; TqSUB-1, T. gondii subtilisin; FP, fluorophosphonate probe; Ab, antibody; BCA, bicinchoninic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ORF, open reading frame; UTR, untranslated region; BFA, Brefeldin A; PI, preimmune rabbit; ER, endoplasmic reticulum.

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Plasmodium (17, 18) and Babesia divergens.3 We therefore hypothesize that these proteases function during B. divergens merozoite invasion, much like they do in Plasmodium falciparum, in two potential ways: the proteolysis of RBC surface and skeletal proteins and the processing of parasite proteins. Many questions remain unanswered regarding the hydrolysis of the RBC surface and cytoskeletal proteins in malaria, including the entire repertoire of enzymes involved, and how parasite proteases come into contact with the RBC proteins. However, recently the “sheddase” that is responsible for the proteolytic shedding of P. falciparum surface proteins during invasion was identified as a membrane-bound subtilisin-like protease called PISUB2 (16).

Proteases in Babesia, in contrast to Plasmodium, have not garnered much research attention besides a single report of a putative cathepsin L-like cysteine protease in B. equi (19). As proteases serve important roles in the metabolism, development, and infectivity of many protozoan parasites they are attractive targets for anti-parasite chemotherapy. A full understanding of the biological role of these enzymes should expedite identification of optimal chemotherapeutic targets and the development of optimal inhibitors of these proteases as anti-parasitic drugs, both for veterinary and human use. In this article, we report the identification of a novel B. diversgens gene, Bdsub-1, that encodes a subtilisin-like serine protease, that is found in the apically situated dense granules, suggesting a function during invasion. This is the first molecular characterization of a protease from any Babesia spp. The B. diversgens-human RBC invasion model that we employed is an accurate reflection of the invasion process in vivo. It also offers several technical advantages over the malaria culture system for studying different aspects of invasion: the high yield of parasites (>70%), the short life cycle of the parasite (8 h) and the higher infectivity and viability of free merozoites that can be obtained in vitro. The development of the B. diversgens human RBC model, offers the ability of directly testing parasite invasion of the RBC, using viable merozoites of B. diversgens, which is not feasible with P. falciparum. Thus, the study of proteases participating in B. diversgens invasion may also advance our understanding of the biology of P. falciparum invasion.

EXPERIMENTAL PROCEDURES

Parasite Propagation—Blood stages cultures of the B. diversgens (Bd Rouen 1986 strain) were maintained in vitro in human A+ RBCs using RPMI 1640 (Invitrogen) medium supplemented with 10% human serum and sodium bicarbonate solution 7.5% (w/v) (Invitrogen). Cells were cultured at 37 °C in a 90% CO2, 5% nitrogen, and 5% oxygen, as previously described (20).

Purification of Free, Viable Merozoites— Cultures were grown to ~60% parasitemia (21). Infected RBCs and culture supernatants were centrifuged at 300 × g for 10 min. The resulting supernatant was first filtered through 5 μm and 1.2 μm reinforced acrylic copolymer membranes (Versapor 3000 and Versapor 1200, Pall Corporation, Ann Arbor, MI). Filtration was performed at 4 °C to avoid merozoite aggregation.

Merozoites were then pelleted at 2000 × g for 10 min. 40 ml of supernatant yielded ~108 merozoites. Free merozoites were fixed with 1% paraformaldehyde for electron microscopy or resuspended in Dulbecco’s phosphate-buffered saline (PBS) (Invitrogen) and sonicated for Western blot. They were resuspended in RPMI 1640 for use in in vitro growth inhibitory assays.

Binding of Biotinylated Fluorophosphonate Probe (FP) to Parasite Proteins—Adapting previous protocols (22), B. diversgens infected cells were lysed with 0.15% saponin (equi-volume), at 37 °C for 10 min, to release parasites, 5 × volume of PBS was added to the suspension and then centrifuged at 3000 rpm for 15 min. Following protocols in other systems (22), the B. diversgens lysate was prepared in 50 mm Tris-HCl buffer, pH 8.0 and protein concentration adjusted to 1 μg/μl. 200 μl of this lysate was used for the reaction with FP-biotin for 30 min at 25 °C. The lysate was incubated for 30 min at 4 °C with one-tenth volume of avidin-agarose beads (Sigma Aldrich) to deplete endogenous avidin-binding proteins. After a brief centrifugation to pellet the beads, the soluble fraction was removed and supplemented with FP-biotin (prepared as a stock reagent in Me3SO) to a final concentration of 2 μM. The reaction was quenched by adding an equal volume of 2× SDS-PAGE buffer before separation on SDS-PAGE and transfer onto nitrocellulose membranes. Blots were blocked in TBS with 1% Tween overnight at 4 °C, and probed with an avidin-horseradish peroxidase conjugate (Bio-Rad, 1:2000 dilution) in TBS-Tween with 1% nonfat dry milk for 30 min at 25 °C. The blot was washed with TBS-Tween three times (10 min/wash), treated with SuperSignal chemiluminescence reagents (Bio-Rad) and exposed to film for 0.1–5 min before development.

Production and Immunoscreening of a B. diversgens cDNA Expression Library—Total RNA was isolated from cultures with ~60% of parasitemia using TRIzol LS Reagent (Invitrogen) and chloroform extraction. The cDNA synthesis and construction of the library was performed by Lofstrand Labs, (Gaithersburg, MD). The cDNA synthesis was carried out using the synthesis kit from Stratagene, La Jolla, CA, the cDNA was column purified to remove species <400 bp, and ligated into EcoRI-XhoI-digested λ Zap Express vector (Stratagene). The primary library had >98% recombinants and contained 2 × 106 pfu. The B. diversgens cDNA library was screened with polyclonal antibodies specific for the PISUB1 mature protease domain (PISUB1m) (23) using standard protocols. Positive clones were purified by the same serum selection procedure and amplified by PCR using T3 and T7 universal primers, and the products were sequenced. DNA sequences and predicted amino acid sequence comparisons were carried out with the GenBankTM + EMBL + DDBJ + PDB and all non-redundant GenBankTM CDS Translations + PDB + SwissProt + PIR + PRF databases, using BLAST and PSI-BLAST algorithm (24), respectively.

Polymerase Chain Reaction—PCR was carried out using TaqDNA polymerase (Promega). Primers for amplification by PCR of the catalytic domain (3’-region of bdsub-1 gene) were BdSub-1-cdF (5’-ACTACAGAGAGGCACCGAAGG-3’) and BdSub-1-cdR (5’-TCACTTTTCTTAGGTGGACAAACC-3’). Primers prepared from bdsub-1 cDNA for amplification by

3 E. Montero, S. Rafiq, S. Heck, and C. Lobo, manuscript in preparation.
PCR and RT-PCR of a genomic sequence of the bdsub-1 gene and the bdsub-1 ORF, using genomic DNA (gDNA) and total RNA (tRNA), respectively, were Bdsub-1F1 (5'-AGCGGCCC-ATACGATGGTTAAGGC-3') and Bdsub-1R1 (5'-ATCTAGATAATATTTCCATCTTCATC-3'). For the PCR, 30 ng of *B. divergens* gDNA was used and 5 μg of tRNA for the RT-PCR. The extension of the 5'-end of the bdsub-1 cDNA was obtained by PCR, using standard PCR protocols and maxipools prepared from aliquots of the amplified *B. divergens* expression library. The primer Bdsub-1R2 (5’-GGAACCTTAACCGCATCCAAG-GCGT-3') that derives from *bdsub-1* cDNA sequence. DNA encoding the putative propeptide of BdSUB-1 was amplified by PCR from the *bdsub-1* cDNA using the oligonucleotide primers, PF1 (5’-ATGGTTAAGCTTGA-3') and PR1 (5’-CTTAAACATCGGCTTCCG-3'). The amplified products were subcloned into Topo TA vector (Invitrogen) for sequencing.

**DNA Sequencing and Predicted Amino Acid Sequence Comparisons (Sequenase) Method Using Custom-Synthesized Primers.** DNA was subcloned into Topo TA vector (Invitrogen) for sequencing. The primer Bdsub-1F1 (5’-GGAACCTTAACCGCATCCAAG-GCGT-3') and Bdsub-1R2 (5’-GGAACCTTAACCGCATCCAAG-GCGT-3') were Bdsub-1F1 (5’-ATGGTTAAGCTTGA-3') and Bdsub-1R1 (5’-TCATTCTGCGCTTCCG-3'). The amplified products were subcloned into Topo TA vector (Invitrogen) for sequencing. The constructs were maintained in the TOP10 Escherichia coli strain (Invitrogen) and then sequenced on both strands. All sequencing reactions were performed by the dideoxynucleotide (Sequenase) method using custom-synthesized primers. DNA sequences and predicted amino acid sequence comparisons were performed with the GenBank™+EMBL+DDBJ+PDB and all non-redundant GenBank™ CDS Translations +PDB+SwissProt+PIR+PRF databases, using BLAST and PSI-BLAST algorithm from the National Center for Biotechnology Information, NCBI (24).

**Southern Blot Analysis—Genomic *B. divergens* DNA was analyzed by high stringency Southern blot (65°C) using a digoxigenin-labeled probe (Roche Applied Science, Indianapolis, IN) designed to encompass the catalytic domain (3'-region of the gene) by PCR using primer pairs BdsUB-1cdF (5’-ACT-ACAGAGAGGCCACCGGAAGGC-3’) and BdsUB-1cdR (5’-TCACCTTTCTAGTTGCAGAAC-3’). *B. divergens* gDNA was digested with a variety of restriction enzymes: Xhol and PstI that do not digest within the gene and Sall, Ndel, HindIII, and BgIII that digest within the gene. 10 μg of each digest was electrophoresed on a 1% agarose gel and transferred to nylon membrane.

After overnight hybridization, the blot was washed twice for 5 min each in 2× SSC and finally for 20 min each in 0.5× SSC at 65°C. Bound probe was detected with disodium-2-chloro-5(4 methoxyspiro[1,2-dioxetane-3.2’-[5-chloro]tricycl[3.3.1.1.3.7] decan]-4-yl)-1-phenyl phosphate (CDP-Star™, Roche Applied Science).

**Protein Expression and Purification and Antiserum Production—DNA encoding Leu24-Lys186 of BdSUB-1 was amplified by PCR from *bdsub-1* cDNA using the oligonucleotide primers, PF1 5’ and PR15’ and cloned into the expression plasmid vector pGEX-6T1 (Amersham Biosciences) according to the manufacturer’s instructions. E. coli strain BL21-gold (DE3) plysS (Stratagene) was transformed with the BdSUB-1p expression plasmid. 250 ml of SOB medium containing 100 μg/ml ampicillin (Sigma Aldrich) was inoculated with 1 ml of fresh overnight culture and grown at 37°C to *A*∞600 = 0.6 prior to induction with 0.2 mM isopropyl-β-d-thiogalactopyranoside. After 4 h of induction, the cells were pelleted and resuspended in B-Per bacterial protein extraction reagent (Pierce) supplemented with a protease inhibitor mixture that contains 4- (2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma Aldrich) for 10 min, the insoluble material was removed by centrifugation and the soluble fraction was used to purify the recombinant protein (called BdSUB-1p). The protein was purified using glutathione-Sepharose 4B (Amersham Biosciences) as described by the manufacturer. Protein concentration was determined by BCA protein assay kit (Pierce).

Polyclonal sera were raised against BdSUB-1p in mice using standard immunization protocols. Briefly, 6–8-week-old male BALB/c mice were injected subcutaneously with 25 μg of BdSUB-1p in 0.1 ml of Freund’s complete adjuvant (Sigma Aldrich). Mice were boosted 14 days later with 25 μg of BdSUB-1p in Freund’s incomplete adjuvant (Sigma Aldrich).

**SDS-PAGE and Western Blot Analysis—**For Western blot assays, cultured parasites were solubilized into three volumes of a saponin buffer (0.15% of saponin in PBS) and incubated at 37°C for 20 min. Samples were washed with PBS by centrifugation for 5 min, and the pellet was resuspended in PBS with a protease inhibitor mixture (Sigma Aldrich) and sonicated. Free merozoite lysates were prepared from sonicates of merozoites suspended in PBS and protease inhibitor mixture (Sigma). After centrifugation, the supernatant was collected and boiled for 5 min in 2× Laemmli sample buffer (Bio-Rad). Samples were run on SDS-PAGE and transferred by electroblotting onto Immuno-Blot™ polivinylidene difluoride membranes (Bio-Rad), which were blocked in blocking solution: Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T) and 3% (w/v) bovine serum albumin (BSA, Sigma Aldrich). Blots were incubated with mouse anti-BdSUB-1p antiserum diluted 1:100 in blocking solution, for 1 h at room temperature. Membranes were then treated with horseradish peroxidase conjugate (Pierce, 1:10,000). Blots were washed with washing buffer (0.5 M NaCl, 0.02 M Tris-HCl and 0.05% Tween-20). Antigen detection was by ECL (SuperSignal® WestPico Chemiluminescent Substrate, Pierce).

**Electron Microscopy—**Free *B. divergens* 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 remaining aldehydes. The fixed merozoites were then dehydrated and embedded in LR-White (Electron Microscopy Sciences, Hatfield, PA). Thin sections of embedded parasites were mounted on parlodion-covered nickel grids, blocked in 2% BSA, and probed with PfSUB1m antibodies overnight at 4°C, washed in buffer containing BSA and Tween 20 and incubated with goat anti-rabbit IgG conjugated to 6-nm gold particles (Electron Microscopy Science) or goat-anti-mouse IgG conjugated to 5-nm gold particles (Amersham Biosciences). After staining with uranyl acetate, sections were observed under a Philips 410 electron microscope (Holland).

**Immunoprecipitation—**Freshly cultured parasites were washed and resuspended in methionine-free medium (RPMI 1640, MP Biomedicals, Inc, Aurora, OH). 200 μCi ml⁻¹ of [35S]methionine/cysteine (PerkinElmer Life Sciences, 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 mixture (Sigma) and centrifuged to collect the supernatant. Lysates were precleared with protein G (Amersham Biosciences) 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 by boiling in sample buffer and run on SDS-PAGE. Gels were stained with Coomassie Blue R-250, fixed with fixing solution (25% isopropyl alcohol, 10% acetic acid) for 30 min and enhanced with Amplify fluoro graphic solution (Amersham Biosciences) for 1 h, dried under vacuum, and exposed to film for autoradiography.

**Monitoring the Effect of Brefeldin A**—The effects of brefeldin A (BFA, Sigma Aldrich) on BdSUB-1 processing during secretory transport was monitored using 35S-radiolabeled B. divergens cultures. The drug was prepared as a stock solution in methanol at 1 mg ml⁻¹ and then added to parasite cultures to a final concentration of 40 μg ml⁻¹ BFA. Methanol was added to the control cultures, and cells were cultured at 37 °C in a 90% CO₂, 5% nitrogen, and 5% oxygen for 1 h. Samples were washed in PBS, lysed in saponin buffer, and analyzed by Western blot using the anti-PfSUB1m antibodies.

**In Vitro Growth Inhibitory Assays (GIA)**—To ensure the antigen specificity of antibody-mediated inhibition, the total preimmune rabbit (PI) and PfSUB1m Igs were purified using protein G-Sepharose (Amersham Biosciences) with IgG binding and elution buffers (Pierce) according to the manufacturer’s recommendation and diazylated with PBS. RPMI supplemented with 10% of human serum and 5% of RBC (6 × 10⁶ RBC) were preincubated at 37 °C in a 90% CO₂, 5% nitrogen, and 5% oxygen for 30 min in 2-cm wells. Purified IgG from PI or PfSUB1m or PBS (control solution) and 2 × 10⁶ free merozoites were added to the prewarmed medium in triplicate wells. The final concentration of Ab in the GIA was 100 μg ml⁻¹ in a 1.2-ml final volume. Samples were incubated at 37 °C with 90% CO₂, 5% nitrogen, 5% oxygen. The invasion efficiency was checked after 24 h and quantitation of parasitemia was performed by counting the total number of intracellular parasites present in 1 × 10⁶ RBC at ×100 magnification using an Eclipse E 600 microscope (Nikon, Tokyo Japan), after Giemsa staining of smears.

**RESULTS**

**Functional Profiling of B. divergens Serine Proteases**—We applied a systems level of analysis as our first approach to the study of serine proteases in B. divergens invasion. Newly developed, potent, selective probes are available for different classes of proteases that allow simultaneous monitoring of the activities of multiple proteases even in crude protein mixture. The chemical FP probe (kind gift from M. Sajjid) specifically directed against the active site of serine proteases (22) identified the expression of all serine proteases present in B. divergens crude extracts by virtue of their catalytic activity. Fig. 1A shows these results. Two dominant bands of protease activity were detected at ~48 and ~75 kDa, which we postulate correspond to two distinct serine proteases. Important controls in this experiment included a parasite extract that was heat-denatured before the addition of the probe (lane 2) and also a RBC extract (not shown) to ensure detected protease bands were of Babesia origin. Information gathered from the use of this active site-directed probe enabled us to assess the number of dominant serine proteases that exist in the intracellular asexual B. divergens parasite.

![FIGURE 1. Identification of serine proteases present in B. divergens extracts.](image)

![FIGURE 2. A PfSUB1m antiserum recognizes B. divergens proteins.](image)
Immunoprecipitation and Western Blot Analysis Reveal Cross-reactive B. divergens Subtilisin Proteases—Because malaria proteases are likely to act on similar substrates like RBC membrane proteins, we decided to use tools derived from P. falciparum serine proteases to identify potential homologues in B. divergens. A similar strategy was used by Miller et al. (15) who used a rabbit polyclonal antiserum specific for the catalytic region of PfSUB1 (PFSUB1m) to clone and characterize TgSUB-1 in T. gondii. Immunoprecipitation and Western blot analysis with the anti-PfSUB1m antibodies revealed cross-reactive B. divergens proteins in the lysates (Fig. 2, A and B). A specific dominant band at ~48 kDa (p48) and a minor band at ~75 kDa (p75) were identified by immunoprecipitation (Fig. 2A). Interestingly, both these correspond approximately to the size of the mature protease (47 kDa) and proprotease (82 kDa) forms of PfSUB1 (23) and to the bands detected by FP-biotin (Fig. 1). Western blot analysis with the same antibodies (Fig. 2B) revealed three BdSUB-1 protein bands p75, p48 and a new band of ~55 kDa, not seen in the immunoprecipitation analysis. These results suggested the presence of B. divergens subtilisin-like proteases similar to those found in P. falciparum.

Cloning of bdsub-1 by Immunoscreening of a cDNA Expression Library.—To identify the putative serine protease observed by immunoprecipitation and to demonstrate that it is indeed a subtilisin; a B. divergens cDNA expression library was prepared and immunoscreened using the anti-PfSUB1m serum. The antibody screening of 1 × 10^7 pfu yielded four immunopositive plaques, each around 2 kb. All 4 clones were identical. One of these clones (bd-1) was sequenced in both directions and further characterized. Complete sequence analysis of the cDNA clone showed a single contiguous sequence 2051-bp long containing an uninterrupted ORF of 1701 bp encoding a protein (BdSUB-1) of 566 amino acids with an estimated molecular mass of 63,683 daltons and an isoelectric point of 8.74. To confirm the bdsub-1 ORF, the 5'-end of the molecule was amplified by a PCR-cloning approach, using standard PCR protocols and maxi pools prepared from aliquots of the amplified library. A fragment of around 1000 bp was amplified, subcloned into Topo TA vector and sequenced. After analysis of the sequence of the PCR product, the 5' cDNA bdsub-1 together with bd-1 clone confirmed that we had the complete open reading frame (1701 bp) and deduced amino acid sequence of BdSUB-1 protein as well as the nucleotide sequence of neighboring upstream (360 bp) region, shown in Fig. 3A. The initial ATG showed a purine in the −3 position upstream and a guanine in the +4 position downstream (25) as well as 10 stop codons before the initial ATG in the 5'-UTR. The entire sequence has been deposited in GenbankTM (accession no. DQ517294).

The bdsub-1 Gene Encodes a Subtilisin-type Serine Protease—PCR amplification of the complete bdsub-1 ORF from total RNA with primers Bdsub-1F1 and Bdsub-1R1, derived from both 5'- and 3'-ends of Bd1, produced a single DNA fragment of 1716 bp. Amplification from gDNA under the same conditions produced a fragment of the same size (Fig. 3B). The coding region of the cDNA and bdsub-1 gene were cloned into Topo TA vector and sequenced. The nucleotide sequences of both the products were identical, suggesting that the bdsub-1 gene contains no introns. Fig. 3A shows a cartoon of the gene highlighting the features of the bdsub-1 gene cDNA and deduced protein product. BdSUB-1 belongs to the subtilisin-like (subtilase, S8) protease superfamily (26). BLAST search of the entire non-degenerate protein databases with the deduced BdSUB-1 protein sequence showed it possesses significant similarity to other known subtilases, particularly those from P. falciparum and P. chabaudi with 30% identity (scores of 183 and 186, respectively) and a lower score of 150 with a subtilisin of the related apicomplexan Toxoplasma gondii. BdSUB-1 also exhibited similar homology to the subtilisin from Neospora caninum (27) (NC-p65) having an identity of 31% with a score of 155. The C-terminal 306 amino acids segment (Ala204–Ile510) was identified as the catalytic domain by PSI-BLAST algorithm (28) with a score of 90.9 and E value of 4e-19. As can be seen from Fig. 4 a high degree of sequence conservation was observed around the catalytic residues, Asp222, His278, and Ser474. Additional conservation was seen at the oxyanion hole residue Asn370. By homology with other subtilases, BdSUB-1 could be synthesized as a pre-pro-protease with a putative signal peptide 23 residues long, predicted by SignalP 3.0. The signal peptide cleavage site most likely lies between Arg and Thr (29). An alignment of BdSUB-1 with sequences from P. falciparum, T. gondii, Pseudomonas sp, and Bacillus sp suggests that the start of the mature protease could be at Asn87 and extend to
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the C-terminal residue Ile566, having a predicted $M_r$ of 42.9 kDa, and the prodomain likely extends from Leu24 to Lys186 with an estimated $M_r$ of 17.5 kDa. Thus, the theoretical molecular mass of the full-length $bdsub-1$ gene product (the preproprotease) is 63,683 Da, and that of the proprotease (i.e., following signal peptide cleavage) is 62,881 Da. Both these sizes are significantly smaller than the molecular mass of the 75 kDa species detected by immunoprecipitation. However, similar results were obtained for both the $P. falciparum$ and $T. gondii$ subtilases, where the predicted molecular mass of PfSUB1 preproprotease (77,874 Da) and proprotease (75,066) and that of TgSUB-1 preproprotease (84,916 Da) were less than the size detected by immunoprecipitation and pulse-chase experiments (15,23).

The $bdsub-1$ Gene Is A Single Copy Gene That Contains No Introns—To establish the copy number of the $bdsub-1$ gene, Southern blot analysis of $B. divergens$ strain was performed. $B. divergens$ gDNA was digested with restriction enzymes Xhol and PstI (which do not cleave within the $bdsub-1$ gene), and SalI, NdeI, HindIII, and BglII (which cleave within the gene). Digests were probed under high stringency conditions with a probe designed to encompass the catalytic domain (1150 bp of the 3'-region of the gene). The hybridization patterns obtained indicated that $bdsub-1$ is a single copy gene (Fig. 5). The $bdsub-1$ cDNA contains no PstI site, and the Southern blot data therefore indicated that the whole locus could be isolated on a single genomic PstI fragment of about 6 kb (Fig. 5, lane 4).

Recombinant Expression of $BdSUB-1$ N-terminal Region in $E. coli$ and Production of Polyclonal Antibodies—The sequence encoding Leu24 to Lys186 of $BdSUB-1$ (BdSUB-1p) was cloned into the expression vector pGEX-6T1 and expressed in $E. coli$ as a GST fusion protein. The predicted mass of the recombinant product (GST-BdSUB-1p) was ~45 kDa. GST-BdSUB-1p was found to be mostly insoluble. Thus, cells were suspended in B-Per lysis solution, to solubilize the recombinant protein. The protein was then purified by affinity chromatography on a column of glutathione-agarose. The purified GST-BdSUB-1p product was analyzed by Western blotting, using an anti-GST monoclonal antibody and used to immunize mice. When the resulting antibodies were used in Western blot assays to probe extracts of free merozoites, a major ~48-kDa protein was recognized. This band thus likely corresponds to the proteolytically active enzyme. Four less abundant bands of 100 kDa (p100), 75 kDa (p75), 25 kDa (p25), and 20 kDa (p20) were also seen (Fig. 6).

$BdSUB-1$ Is Localized to Dense Granules—To localize $BdSUB-1$ within the merozoite, immunoelectron microscopic analysis was carried out on sections of free $B. divergens$ merozoites, using the anti-PfSUB1m antibodies. As can be seen in Fig. 7, discrete antibody reactivity was observed with circular, electron dense organelles with the morphological characteristics of merozoite dense granules (labeled with arrows in Fig. 7). Immunoreactivity was observed only in the granules situated toward to the apical end of the merozoites. Moreover, p48 was the major $bdsub-1$ gene product found in Babesia merozoite extracts by Western blot and immunoprecipitation and is potentially the final product of $BdSUB-1$ processing in the parasite. If this is so, p48 is the protease species that concentrates in the merozoite dense granules. Thus, $BdSUB-1$ appears to have the same location in merozoites as PfSUB1, indicating that it may be a true PfSUB1 homolog. Similar localization in the dense granules was obtained using the anti-BdSUB-1 antibodies, although the staining was not as strong (data not shown).
BdSUB-1 Undergoes Post-translational Processing during Secretory Transport—Subtilases are synthesized as enzymatically inactivezymogens, activation of which invariably requires one or more proteolytic cleavages of the precursor. In order to examine whether BdSUB-1 undergoes intracellular post-translational processing steps within the parasite secretory pathway, we employed BFA, which blocks secretory transport of proteins from the endoplasmic reticulum (ER) to the Golgi apparatus. Western blot analysis was performed on parasite lysates that were obtained from cultures grown in the presence or absence of BFA, using anti-PfSUB1m antibodies. Fig. 8 shows the presence of relatively large amounts of a 55-kDa protein (p55) (lane 2), which was not detected in cultures grown in the absence of BFA.

The 48 kDa end product of BdSUB-1 processing of size (p48) was seen in both lanes 1 and 2. This suggests that p55 is the BdSub-1 product that accumulated in the ER in the presence of BFA, and p48 in lane 1, represents the already processed BdSUB-1, present in the culture before the use of BFA. Thus, as we propose in Fig. 3, it appears that BdSUB-1 is initially synthesized as a large precursor protein of ~78 kDa, and cleavage of the signal peptide results in the formation of p75, which is the largest detectable protein that we see in our assays. This p75 is then processed to p55, which then gets converted to p48 in the Golgi. Thus, use of BFA interferes with the conversion of p55 to p48, resulting in an accumulation of p55 in BFA-treated cultures (Fig. 8, lane 2).

Purified anti-PfSUB1m Antibodies Inhibit in Vitro Invasion of the Parasite—A B. divergens in vitro inhibition of invasion assay using free merozoites and IgG purified from PfSUB1m antiserum was carried out. Giemsa-stained thin blood smears were prepared and the parasitic growth was monitored after 8 h. Potent inhibition of parasite invasion was observed in these cultures in the presence of both anti-PfSUB1m serum and antibodies purified from this serum (Table 1). The percent of inhibition of invasion of merozoites in the PfSUB1m IgG group was significantly higher than the control (without antibodies) and the IgG-purified antibodies from preimmune samples (58% inhibition, with a statistically significant p value of 2 × 10⁻⁴). On examination of Giemsa smears of parasites grown in the presence of purified IgG from anti-PfSUB1m serum, a large number of free extra-erythrocytic merozoites were observed by light microscopy (Fig. 9A). This led us to speculate that the antibodies were mediating their inhibition at the time of invasion. Because BdSUB-1 is not localized on the surface of the merozoites, we hypothesized that it may be released into the culture supernatant, thus permitting interaction of the antibodies with the subtilisin. To confirm this, we analyzed radio-
labeled culture supernatants for the presence of BdSUB-1. Two immunoreactive bands can be clearly seen, corresponding to the 48-kDa active protease and a lower molecular weight band, which might represent further processing or degradation of the protein. (Fig. 9B). These results indicate that the protease is secreted from the merozoite at or around the point of invasion, suggesting a role in invasion.

**DISCUSSION**

Babesiosis is fast becoming an important parasitosis because of two factors: (i) that *Babesia* is now recognized as a zoonotic parasite (9), with humans acquiring infections from mammalian animal reservoirs; and (ii) that *Babesia* represents a potential threat to the blood supply for transfusions because asymptomatic infections in humans are common and the spread of parasite via blood transfusions has been frequently reported (31). Invasion of erythrocytes is an integral part of the *Babesia* life cycle. The process of invasion by apicomplexan parasites, is a carefully coordinated process, involving the regulated release of specialized secretory organelles. Several lines of evidence suggest that proteases are critical for the assembly and trafficking of organelar content proteins (32, 33). Further, invasion is accompanied by cleavage and shedding of secreted proteins as host cell invasion occurs. Serine protease inhibitors block invasion in *Plasmodium* (17), and studies from our laboratory show similar inhibition in *B. divergens*. Proteases may serve a vital role in the infectivity of the *Babesia* merozoites by hydrolyzing the erythrocyte surface proteins and the complex erythrocyte cytoskeletal network to permit movement of the parasite into and out of the erythrocyte. By virtue of their function, we hypothesized that they should be conserved in structure among different hemoparasites, particularly between *Plasmodium* and *Babesia*. Because *P. falciparum* and *B. divergens* are harbored by the same host cell, the human RBC, tools derived from studies of the malaria invasion machinery were used to obtain analogous information in *Babesia*. We anticipate that such data will in the long term represent an excellent opportunity to compare and contrast mechanisms of merozoite invasion between these species.

**TABLE 1**

| B. *divergens* samples          | 8 h invasion | % Invasion relative to the control |
|---------------------------------|--------------|-----------------------------------|
|                                 | Mean ± S.D.  |                                    |
| PBS                             | 1.8 ± 0.2    | 100                                |
| PI-purified rabbit Ab           | 1.8 ± 0.3    | 100                                |
| PI-purified rabbit Ab           | 0.6 ± 0.05   | 42                                 |

**FIGURE 8.** Analysis of BdSUB-1 processing in parasites in the presence of brefeldin A. Parasites were treated with 40 μg/ml of BFA in methanol (lane 1) or methanol only (lane 2) for 1 h at 37°C. Samples were analyzed by Western blot using the PfSUB1m antibodies. Positions of molecular mass markers are shown. BFA blocked the secretory transport of BdSUB-1 from the ER to the Golgi apparatus, resulting in the accumulation of p55.

**FIGURE 9.** Purified anti-PFSUB1m antibodies inhibit in vitro invasion of the parasite. Preincubation of *B. divergens* free merozoites with purified PFSUB1m antibodies (100 μg) reduced the efficiency of invasion of erythrocytes by 58%. A, J, Giemsa stained thin blood smears show normal parasite invasion after 8 h. In lane 1, a high number of free extra-erythrocytic merozoites were visualized in the Giemsa smears of *B. divergens* cultures treated with anti-PFSUB1m IgG, after 8 h. B, BdSUB1 is secreted into culture supernatants: Parasites were biosynthetically radiolabeled with [35S]methionine/cysteine for 9 h, after which the supernatant was analyzed by immunoprecipitation. Lane 1, preimmune rabbit sera. Lane 2, PFSUB1m antibodies recognize the 48-kDa active protease and a lower molecular weight fragment in the culture supernatant.
two parasites. Recent studies from our laboratory, using invasion assays into phenotypically distinct RBCs have shown that *B. divergens* uses neuraminidase- and trypsin-sensitive receptors, of which glycophorins A and B are the prominent ones, for invasion, similar to *P. falciparum* (34). Data presented in this article highlight additional similarities in structural and functional aspects of the proteolytic invasion machinery.

We took advantage of a newly developed functional proteomics tool in the form of chemical probes specifically directed against the active site of serine proteases (22). This “chemical genetics” approach has the advantage of obtaining detailed functional information without the need for prior purification and identification of target proteases. Although relatively new, it has already been widely used to detect selective protease activity in a wide variety of systems, including parasites. FP-biotin was used to profile and identify serine proteases in complex crude lysates of the parasite, by virtue of their catalytic activity. Two bands corresponding to potent serine protease activity were detected at ~45 (p48) and ~75 kDa, respectively (Fig. 1, lane 1). Interestingly, we saw subtilisin bands of similar molecular weight in immunoprecipitation experiments with anti-PfsSUB1m antibodies (Fig. 2A) and anti-PfsSUB2 antibodies (data not shown) probably corresponding to the mature, active enzymes recognized by FP-biotin probe. Using these antibodies against PfsSUB1, we have successfully cloned the gene encoding the corresponding homologous enzyme in *B. divergens* (*Bdsub-1*). Thus, we have identified the first known subtilisin protease of this species.

Although we have not yet been able to demonstrate any proteolytic activity associated with BdSUB-1, the results presented in this article clearly show that it is likely to be proteolytically active during merozoite invasion. BdSUB-1 has a clear homology, overall and within the catalytic domain, with the apicomplexan enzymes PfsSUB1 and TgSUB-1 and other subtilisins (identity of ~30%). Notably, BdSUB-1 possesses all of the typical features required of active subtilisins (35), including the catalytic triad residues essential for proteolytic activity (Asp222, His278, and Ser274) and a glycine residue (Gly472) two positions N-terminal to the active site serine. BdSUB-1, also possesses an asparagine (Asn370) at the position of the oxyanion hole residue. Other typical features were found including a set of seven cysteine residues within the putative catalytic domain, responsible in a large number of subtilisases for disulfide bond formation (35).

The post-translational processing that we have detailed for BdSUB-1 lends support to the idea that it is a functional protease. Subtilisases are synthesized as zymogens, which consist minimally of a signal peptide, a propeptide domain and a catalytic domain. By homology with other subtilisases, BdSUB-1 is predicted to undergo at least one additional processing event, involving limited proteolytic activity at the signal peptide cleavage site. A second proteolytic cleavage site is present approximately two positions N-terminal to the active site serine.

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A Subtilisin Protease from Babesia divergens

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