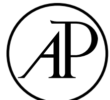

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Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) calreticulin ☆

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Abstract

We report the cloning, sequence characterization and expression analysis of a calreticulin (CRT) coding cDNA of *Boophilus microplus*. CRT is a calcium-binding protein involved in multiple cell functions and possibly implicated in parasites host immune system evasion. The CRT cDNA sequence and its molecular characterization are described. Sequence similarity and phylogenetic analyses indicate a close relationship to other arthropod CRT sequences. The CRT cDNA was also expressed in a procariotic system and the recombinant protein (rBmCRT) was used to raise antibodies in a rabbit. Expression analyses of the corresponding gene in different developmental stages and tissues were performed by RT-PCR and Western-blot, which indicated a ubiquitous expression of the *B. microplus* calreticulin gene and demonstrated its presence in saliva. Sera of tick-infested bovines suggested that this protein may not be able to induce an IgG-based humoral response in its natural host.

Index Descriptors and Abbreviations: *Boophilus microplus*; Tick; Gene expression; Parasite–host relationship; CRT, calreticulin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; pfu, plaque forming units; ER, endoplasmic reticulum; IgG, immunoglobulin G. © 2002 Elsevier Science (USA). All rights reserved.

1. Introduction

Ticks are blood-sucking arthropods that infest a wide array of species (Sauer et al., 1995), including humans and some animals of economic importance, and cause important losses to livestock production (Bowman et al., 1996). One-host ixodidae ticks, in particular, expend most of their life feeding on the host, therefore they must be able to deal with all problems of haemostatic and immunologic origin the vertebrate blood may bring to a parasite (Ribeiro, 1989; Sauer et al., 1995). Salivary secretions are well recognized to perform such modulatory events in the tick–host relationship (Ribeiro, 1989, 1995; Wikel, 1999), but the purification and further characterization of these activities are many times unfeasible due to the small amounts of saliva that are available from ticks. An alternative approach would be the identification of tick salivary genes in bacterial expression systems and search for their biological functions using recombinant proteins.

Calreticulin (CRT) is a calcium-binding protein, known to perform several functions in mammals (Michalak et al., 1999). CRT is also secreted by ticks into their hosts (Jaworski et al., 1995) and its involvement in host immune system modulation has been suggested (Jaworski et al., 1995; Kovacs et al., 1998). The protein is divided into three domains: a N-terminal domain

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(N-domain), which is the most conserved domain among all CRTs (Michalak et al., 1999); an internal domain (P-domain), which binds Ca^{+2} with high affinity (Baksh and Michalak, 1991); and a C-terminal domain (C-domain), which is highly acidic and exhibits a high-capacity of Ca^{+2} binding (Baksh and Michalak, 1991).

Boophilus microplus is a one-host tick that usually infests cattle and is responsible for economic losses ranging around billions of dollars per year (Bowman et al., 1996). However, as to how this parasitic relationship is accomplished, very little is known. The characterization of salivary gland-associated molecules may improve our knowledge on the mechanisms involved in tick–host interaction and how they could be inhibited. We report here the isolation, sequence characterization, and expression analysis of a salivary calreticulin coding cDNA of *B. microplus*. We have also tested the immunogenicity of the corresponding protein (BmCRT) under infestations, and by immunization with the recombinant protein.

2. Material and methods

2.1. Ticks and harvest of saliva

B. microplus females, eggs, and larvae (Porto Alegre strain) were maintained in an incubator at 28 °C and 85% relative humidity, and their parasitic life was completed in calves, housed in individual pens on slatted floors. Partially engorged adult female ticks were obtained by direct detachment from the calves, kept in a wet chamber, and salivation was induced by injection of 5 μl of 2% pilocarpine solution. Saliva was then collected for a period of 2 h directly from tick mouthparts and stored at −70 °C until use.

2.2. Antigen preparation

Fully and partially engorged female ticks were washed with phosphate-buffered saline pH 7.2 (PBS) plus 500 IU penicillin/ml. The dorsal surface was dissected with a scalped blade. Salivary glands, guts, ovaries, and fat bodies were separated with fine-tipped forceps and washed in PBS. These materials were kept frozen at −70 °C until use.

The frozen tissues were thawed and protein extracts prepared according to Da Silva Vaz Jr. et al. (1994). The protein concentrations of the extracts were measured using the Bradford method (1976) with bovine serum albumin as standard.

2.3. Antibody production

Antibodies against salivary gland were raised in a rabbit by inoculation of 100 μg salivary gland extract emulsified with an equal volume of Freund's complete adjuvant. Three additional boosters were given every three weeks with 100 μg of antigen emulsified in Freund's incomplete adjuvant. Fifteen days after the last booster serum was collected.

2.4. Synthesis and screening of the salivary gland cDNA library

An unidirectional cDNA library was synthesized from salivary gland poly$(A)^+$ RNA of partially engorged adult females using the UNIZAP vector (Stratagene), according to the instructions of the manufacturer. The poly$(A)^+$ RNA was obtained using the Micro-Fast Track Kit (Invitrogen). The immunological screening was performed using the anti-salivary gland serum. Nine thousand recombinant cDNA clones were screened on nitrocellulose membranes (Schleicher & Schüll) resulting in a single positive CRT-similar clone, named Bmsg1. Membranes were blocked with blotto (5% cow non-fat dry milk in PBS) for 1 h at room temperature, and then incubated with anti-salivary gland antibodies at room temperature for 18 h. After three washes with blotto, goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma) diluted 1:5000 in blotto was incubated for 1 h at room temperature. After three washes with PBS and once with development buffer (5 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris, pH 9.5), membranes were stained with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT).

The Bmsg1 cDNA was used to screen the same cDNA library in order to obtain the full cDNA coding region of BmCRT. Nine thousand recombinant plaque forming units (pfu) were screened on nitrocellulose membranes (Schleicher & Schüll) using the Nucleic Acids ECL Kit (Amersham–Pharmacia), according to the instructions of the manufacturer.

All positive clones isolated were excised into the pBluescript II (Stratagene) plasmid and their inserts analyzed by sequencing and digestion with restriction enzymes.

2.5. DNA sequencing and analysis

DNA sequencing of all cDNAs isolated was performed on an ABI-PRISM 377 automated DNA sequencer (Perkin–Elmer) at Molecular Genetics Facility, University of Georgia, Athens, Georgia, USA.

The FASTA algorithm (Pearson and Lipman, 1988) was used to analyze the nucleotide and deduced amino acid sequence homologies with previously reported sequences within databases. Multiple alignment of CRT sequences was performed with CLUSTALW (Thompson et al., 1994).

2.6. Phylogenetic analysis

CRT sequences were aligned with the BmCRT deduced protein sequence using the CLUSTALW program

(Thompson et al., 1994). An unrooted neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was created with the proportion of pair-wise nucleotide differences (p-distances) using the MEGA program (Kumar et al., 1993). Bootstrap support was assessed using 500 replicates. The GenBank accession numbers are: *Amblyomma americanum*, AAC79094; *Aplysia californica*, JH0795; *Arabidopsis thaliana*, O04151; *Beta vulgaris*, O81919; *Bos taurus* (bovine brain isoform 2), S36799; *Caenorhabditis elegans*, P27798; *Chlamydomonas reinhardtii*, Q9STD3;*Danio rerio*, AAF13700; *Dictyostelium discoideum*, Q23858; *Dirofilaria immitis*, AAD03405; *Drosophila melanogaster* P29413; *Euglena gracilis*, Q9ZNY3; *Homo sapiens* (human), NP_004334; *Leishmania donovani*, U49191.1; *Litomosoides sigmodontis*, CAA04877; *Mus musculus* (mouse), NP_031617; *Necator americanus*, CAA07254; *Onchocerca volvulus*, P11012; *Oryctolagus cuniculus* (rabbit), P15253; *Oryza sativa*, Q9SLY8; *Prunus armeniaca*, Q9XF98; *Rana rugosa*, S71343; *Rattus norvegicus* (rat), NP_071794; *Ricinus communis*, P93508; *Schistosoma japonicum*, AAC00515; *Schistosoma mansoni*, Q06814; *Strongylocentrotus purpuratus*, AAD55725; *Trypanosoma cruzi*, AAD22175; *Tritrichomonas suis*, CAB92410; *Zea mays*, S58170.

2.7. Construction of a plasmid expressing the mature BmCRT

The coding region of mature BmCRT was subcloned into the pGEX-4T1 vector (Amersham–Pharmacia), using the restriction sites of *Bst*Z17 (nucleotides 79–84; indicated in Fig. 1) and *Xho* I (pBluescript). As there is a site for *Xho*I within Bmcrt, a partial cleavage was performed to obtain the correct fragment to be cloned, which produces a recombinant protein that lacks the signal peptide plus four amino acids of the mature protein (see Fig. 1). The fragment was cloned within the sites of *Xho*I and *Sma*I of pGEX-4T1. Correct cloning was confirmed by sequencing, and the recombinant plasmid named pGEX-CRT.

The glutathione–Sepharose 4B (Amersham–Pharmacia) affinity chromatography was used to purify rBmCRT. Lysogens of BL21/pGEX-CRT were prepared after growth in Luria–Bertani medium. Recombinant protein expression was induced with IPTG 0.1 mM. Cell pellet from 2000 ml culture were suspended in 20 ml of PBS and frozen at −70 °C. Cells were thawed and disrupted in a French press. Triton X-100 was added to the supernatant to a final concentration of 1%. The supernatant was then loaded on the column that had been equilibrated and washed with buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and the fusion protein was then incubated with thrombin overnight at 23.5 °C. The GST portion and the remaining fusion protein in the column were eluted with buffer B (50 mM Tris–HCl + 10 mM glutathione, pH 8.0). Protein purity was monitored by a 10% SDS–PAGE (Laemmli, 1970) stained with Coomassie blue G-250.

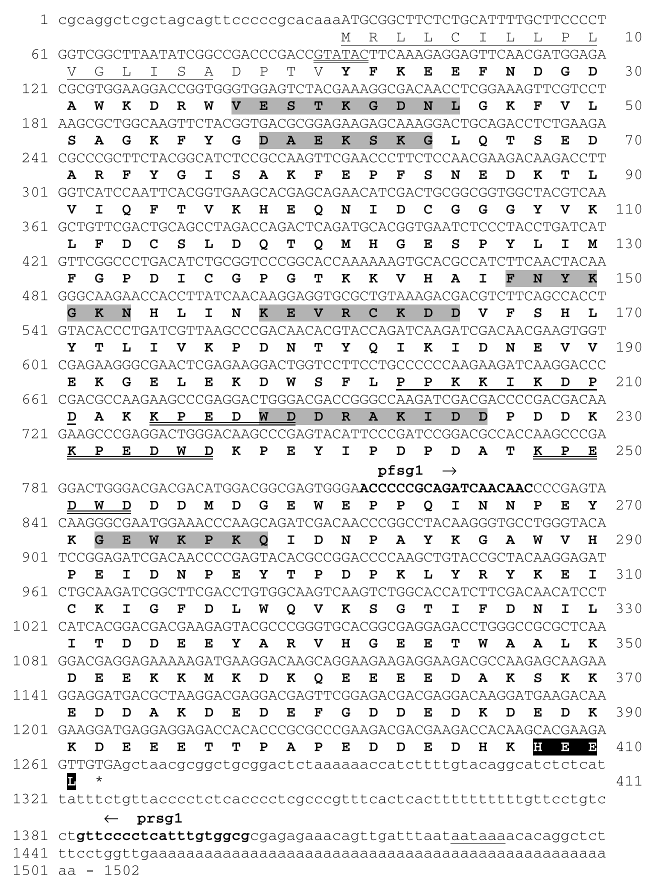

Fig. 1. DNA sequence, and predicted translation of Bmcrt. Along the nucleotide sequence are shown: the ORF (uppercase); the untranslated regions (lowercase); the restriction site of *Bst*Z17 used for cloning (double underlined); the regions used for the synthesis of primers pfsg1 and prsg1 (in bold); the stop codon (asterisk); and the polyadenylation signal (underlined). Along the amino acid sequence are shown: the signal peptide (underlined); the sequence of the recombinant protein (in bold); the potential C1q-binding sites (light shaded); the nuclear localization signal (underlined); the three CRT family repeat-motif signature patterns (double underlined); and the ER retention signal (dark shaded).

2.8. RNA poly(A)$^+$ purification and RT-PCR

RNA poly(A)$^+$ was purified from *B. microplus* tissues and bovine blood collected with citrate, and stored at −70 °C. The RNA poly(A)$^+$ purification was performed with the Quick Prep Micro mRNA Purification Kit (Amersham–Pharmacia), as described by the manufacturer. Five hundred ng of RNA poly(A)$^+$ were submitted to reverse transcription (RT) at 37 °C in the presence of oligo-dT (Amersham–Pharmacia) and M-MLV Reverse Transcriptase (Gibco-Life Technologies) also according to the instructions of the manufacturer. PCR amplifications were performed using 1/20 of the RT reaction, 10 pmol of each primer (pfsg1 and prsg1) and 2.5 U of

Taq DNA polimerase (Cenbiot) in a final volume of 50 μl. Samples were denatured for 10 min at 94 °C and amplification was achieved through 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C, with a final extension cycle of 30 s at 52 °C and 10 min at 72 °C. Negative controls for the RT reactions and PCR amplifications were always included into the assay. Amplicons were visualized by agarose gel electrophoresis and ethidium bromide staining.

2.9. Immunogenicity of rBmCRT

rBmCRT was further purified by 10% SDS–PAGE electrophoresis and one rabbit was subcutaneously inoculated with four doses of approximately 100 μg of protein emulsified in Freund's incomplete adjuvant. Fifteen days after the last booster serum was collected. One bovine was also immunized with the same rBmCRT preparation. In the initial four inoculations 150 μg of rBmCRT were used; in the following boosters the amount of protein were raised to 250 μg (fifth booster), 350 μg (sixth booster), and 400 μg (seventh and eighth boosters).

Six bovines were artificially infested repeatedly 12 times: 6 times with 18,000 *B. microplus* larvae followed by 6 times with 800 larvae, and sera were collected after each infestation. *Rhippicephalus sanguineus* infested and non-infested dog sera were obtained at the Veterinary Hospital of Universidade Federal do Rio Grande do Sul.

2.10. Western-blot

For Western-blot analysis, tissue extracts and purified rBmCRT were resuspended in sample buffer containing 2% SDS, 250 mM Tris, pH 6.8, 0.025% bromophenol blue, 5% glycerol, 10% β-mercaptoethanol, and 5 M urea, separated in SDS–PAGE 10% gel electrophoresis and transferred to nitrocellulose at 70 V for 1 h at 4 °C in 12 mM carbonate buffer pH 9.9 (Dunn, 1986). The nitrocellulose sheet was blocked with blotto for 2 h at room temperature. In the Western-blot shown in Fig. 5, the anti-rBmCRT rabbit serum (1:2000) was incubated in blotto overnight at 4 °C, and the secondary antibody conjugated to alkaline phosphatase and development procedure was the same used in the immunological screening.

In the Western-blots shown in Fig. 6 the protocol for the SDS–PAGE, protein transfer to nitrocellulose and blocking were the same as described above. In Fig. 6a and c the antigen used was rBmCRT at a concentration of 12 μg per nitrocellulose strip (strips of 4 mm; 30 μg/cm), and in Fig. 6b the antigen used was a partially engorged salivary glands extract at a concentration of 44 μg per strip (110 μg/cm). The *B. microplus* infested bovines sera were diluted 1:20, the *R. sanguineus* infested dog sera 1:50 and the anti-rBmCRT rabbit serum 1:400. Prior to the overnight incubation at 4° C with the antigens, all sera were diluted in an *E. coli* BL21 strain lysate expressing the pGEX-4T1 vector and incubated for 2 h at room temperature for absorption of anti-*E. coli* and anti-vector derived protein antibodies. Preparation of the *E. coli* BL21 strain lysate was performed according to Rott et al. (2000). The secondary antibodies used were conjugated to peroxidase (anti-bovine IgG, Sigma, diluted 1:2000; anti-dog IgG, Sigma, diluted 1:2000), and after the 1 h incubation three washes with PBS were performed and the development buffer (5 mg 3,3′-diaminobenzidine in 30 ml PBS plus 150 μl H_2O_2 30% and 100 μl $CoCl_2$ 1%) was added.

3. Results

3.1. Isolation of the cDNA clones

A cDNA clone (Bmsg1) was obtained by immunological screening from a salivary gland library using a rabbit anti-salivary gland serum (as described in Section 2). The Bmsg1 sequence was determined, which encodes a 1 kbp cDNA fragment with a 254 amino acids ORF with high similarity to CRT sequences. As no ATG codon that could code for an initiating methionine was evident and a N-terminal region was apparently missing (deduced from sequence comparison), Bmsg1 DNA was then used to screen the same library in order to obtain a full-length coding sequence cDNA. Twenty-four positive clones were obtained out of 9000 recombinant pfu, and four of them were sequenced at their ends based on insert size and restriction endonucleases digestion pattern. All of them presented the same sequence, which were identical to Bmsg1 at the C-terminus. One of them, named Bmcrt, was then fully sequenced and its deduced protein sequence also was shown to be highly similar to CRTs from other organisms (Fig. 2), and to contain a putative full-length coding sequence. Fig. 1 shows the 1502 bp sequence of Bmcrt and its deduced amino acid sequence.

3.2. Sequence, similarity, and phylogenetic analysis

Bmcrt possesses an ORF of 1233 bp that encodes a protein of 47.7 kDa. A probable signal peptide of 16 predominantly hydrophobic amino acids is present at the N-terminus, which begins at a putative initiation codon (Fig. 1). The presence of the signal peptide was corroborated by the N-terminal microsequencing of a native salivary BmCRT-similar protein, which showed that the mature protein began at the aspartic residue 17 (Carlos Termignoni, personal communication). The predicted molecular mass and p*I* for the mature protein are 46 kDa and 4.48, respectively. BmCRT possesses six

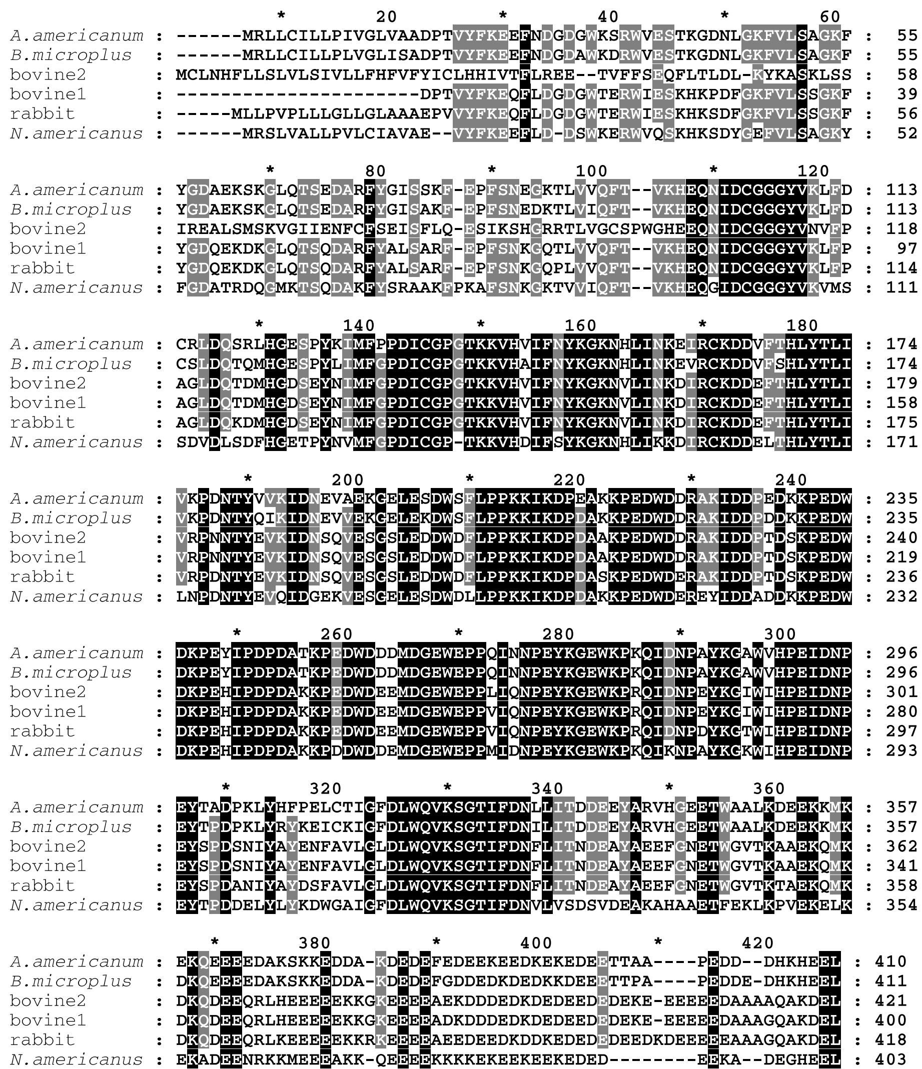

Fig. 2. Multiple alignment of the deduced amino acid sequence of Bmcrt and other CRT sequences. GenBank accession numbers: *Amblyomma americanum*, U07708; Bmcrt, AF420211; bovine isoform 1 (*Bos taurus*), P52193; bovine isoform 2 (*B. taurus*), L13462; rabbit (*Oryctolagus cuniculus*), P15253; *Necator americanus*, AJ006790. Dark shading shows total identity and light shading shows residues conserved in five sequences.

potential human-like C1q-binding regions, at positions 37–45, 58–64, 147–153, 158–165, 218–225, and 272-278. A consensus nuclear localization signal is found at position 202–211 (Fig. 1). Three CRT family repeat-motif signature patterns (IXDXDXKKPEDWD; Michalak et al., 1992) are present at residues 207–219, 225–236, and 241–253, with an alteration from K to T in the third repeat. The last four residues comprise a modified endoplasmic reticulum (ER) retention signal (HEEL). A 235 nucleotides long 3′ untranslated region follows the termination codon, with a putative polyadenylation signal positioned 21 bp from the poly-A tail.

Based on sequence similarity, the three domains of BmCRT were deduced: N-terminal domain (N, residues 17–196), the internal domain (P, residues 197–306), and the C-terminal domain (C, residues 307–411). Fig. 2 shows the alignment of BmCRT deduced sequence with CRTs of bovine, rabbit, the tick *A. americanum*, and the hookworm *N. americanus*. It can be seen that BmCRT shares the high conservation observed among the CRT sequences, mainly within the N and P-domains. The C-domain is more variable, but a high content of acidic residues is conserved in all sequences. Two isoform sequences of bovine CRT were included in the alignment, and the isoform 2 (bovine 2) showed a N-terminal sequence that diverges from all other CRTs. The identity shared by BmCRT and mammalian CRTs (mouse, rat, human, rabbit, bovine-isoform1) lies in the range of 67–70%, while identities with CRTs of the other arthropods *A. americanum* and *D. melanogaster* are, respectively, of 90% and 67% (data not shown). The identities shared with other parasites are smaller, as can be seen with *N. americanus* (63%) and *S. japonicum* (52%) (data not shown).

The neighbor-joining phylogeny constructed with CRT sequences is shown in Fig. 3. Considering the generally high bootstrap values, a monophyletic origin

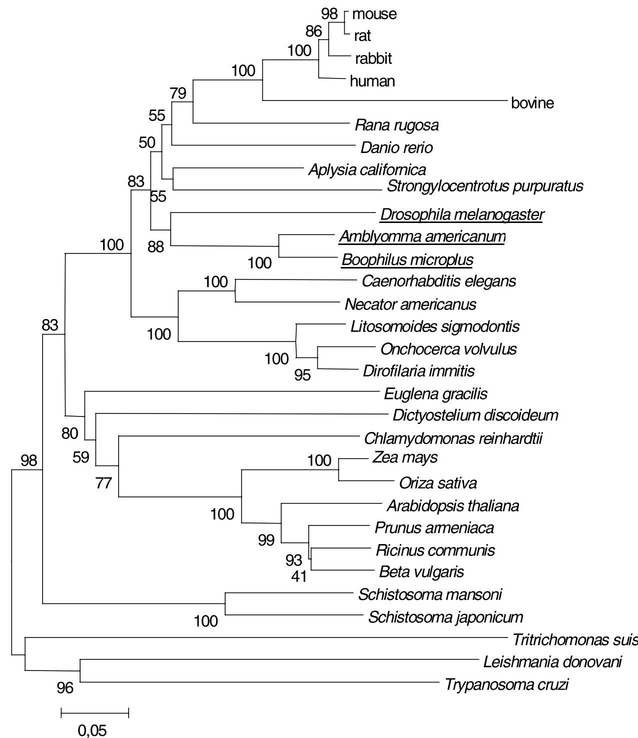

Fig. 3. Dendrogram of CRT sequences. The scale bar indicates 0.05 substitutions per site. Bootstrap proportions are indicated at branches. Concerning the bovine sequences, only bovine CRT isoform 2 was used because complete sequence was not available for isoform 1. Arthropod sequences are underlined.

of the vegetal CRTs can be inferred. Within the animal kingdom CRTs seem to be paraphyletic, due to the independent and intermediate positions of the *Schistosoma* spp. and protozoan clades. However, the tree strongly supports a common origin of all arthropod CRT sequences.

3.3. RT-PCR analysis of Bmcrt expression

The expression analysis of the Bmcrt gene was performed by RT-PCR using one primer annealing in the untranslated 3′ end region of the gene (prsg1), to ensure Bmcrt specific amplification, and a primer annealing within the coding region (pfsg1) (Fig. 1), generating an amplicon of 588 bp. All tissues and developmental stages tested showed the predicted fragment amplification (Fig. 4), indicating that Bmcrt is ubiquitously expressed in *B. microplus*. The same fragment size was obtained when Bmcrt was amplified from genomic DNA, indicating that this region of the gene does not possess introns, or if it does, they must be small.

3.4. Identification of BmCRT within tick tissues and developmental stages

The Bmcrt cDNA was subcloned into the pGEX-4T1 vector (Amersham–Pharmacia) and expressed in *E. coli*.

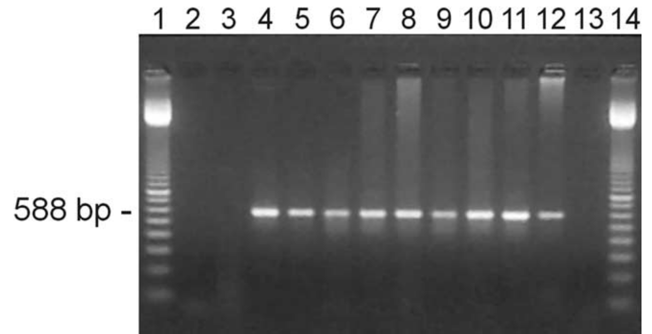

Fig. 4. Amplicons of BmCRT generated by RT-PCR from RNA poly(A)$^+$ of different tissues/developmental stages. Lanes 1 and 14, molecular mass standards (100 bp ladder, Amersham–Pharmacia); lane 2, a representative of the negative controls of reverse transcriptase reactions; lane 3, negative control of the PCR reactions; lane 4, cloned DNA (positive control); lane 5, genomic DNA; lane 6, larvae; lane 7, ovaries (late in development) and lane 8, ovaries (early in development) from partially engorged females; lane 9, fat bodies from fully engorged females; lane 10, salivary glands from partially engorged females; lanes 11, guts from partially engorged females and 12, from fully engorged females; lane 13, bovine blood (negative control). Samples were submitted to electrophoresis on a 2% agarose gel and stained with ethidium bromide.

rBmCRT, cleaved out from the fusion protein, was used to raise a hyperimmune serum on a rabbit.

The anti-rBmCRT serum was used in a Western-blot analysis of tick tissues/developmental stages, as can be seen in Fig. 5. The predicted band corresponding to BmCRT was present in all samples tested, corroborating the data from the RT-PCR. The presence of BmCRT in the saliva is also shown, indicating that it is secreted into the host while feeding.

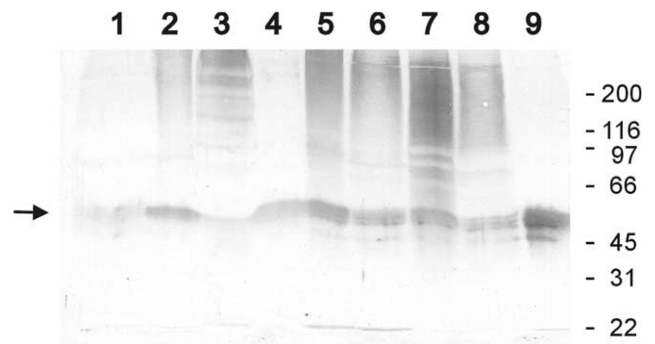

Fig. 5. Western-blot of *B. microplus* tissues and developmental stages extracts, saliva and purified rBmCRT. Samples were run on a 10% SDS–PAGE gel and transferred to a nitrocellulose membrane. Anti-rBmCRT rabbit serum was used as probe. Lane 1, saliva from partially engorged females (75 μg); lane 2, partially engorged female salivary gland extract (36 μg); lane 3, ovary extract (61 μg); lane 4, partially engorged female gut extract (180 μg); lane 5, fully engorged female fat body extract (23 μg); lane 6, total adult males extract (40 μg); lane 7, total non-engorged adult female extract (15 μg); lane 8, larvae extract (96 μg); lane 9, purified rBmCRT (2 μg). Numbers at left are molecular weight standards expressed in kDa (Broad—Bio-Rad). An arrow indicates the 55–60 kDa bands representative of BmCRT and rBmCRT. A replicate was performed using pre-immune serum as control (data not shown).

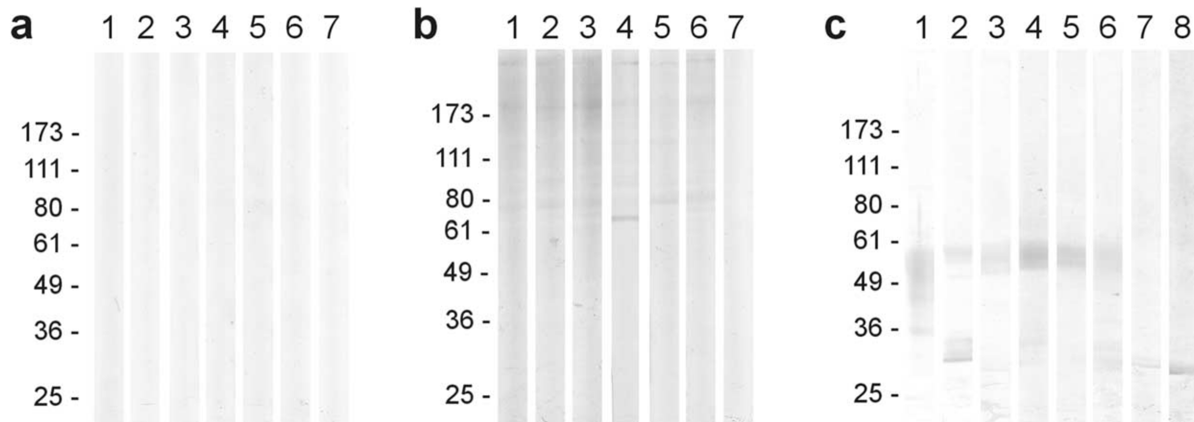

Fig. 6. Western-blot analysis of tick infested animals sera against rBmCRT (a) and (c) and partially engorged salivary gland extract (b). (a) Lanes 1–6, sera of bovines; lane 7, secondary antibody conjugate control (without serum). (b) Lanes 1–6, sera of bovines; lane 7, secondary antibody conjugate control (without serum). Replicates were performed using sera from the bovines taken before infestations as a negative control (data not shown). (c) Lane 1, anti-rBmCRT rabbit serum; lanes 2–6, sera from dogs naturally infested with *R. sanguineus*; lanes 7–8, sera from non-infested dogs. Molecular weight standards are expressed in kDa (Gibco-Life Technologies).

3.5. Immunogenicity of BmCRT

In order to evaluate if natural hosts produce antibodies against tick secreted BmCRT, sera from six bovines collected after eight consecutive artificial infestations were tested for the presence of anti-rBmCRT antibodies by Western-blot assay. The six sera did not recognize rBmCRT (Fig. 6a), but they recognized numerous bands when a salivary gland extract was used as antigen, and none of them corresponded to BmCRT (Fig. 6b). The same results were found in similar experiments using a more sensitive development system (alkaline phosphatase conjugated to the secondary antibody) with the same sera, with sera from the same animals after different numbers of infestations, and from naturally infested bovines (data not shown). We have also made eight intramuscular immunizations on a bovine with rBmCRT (using 150–400 μg in each inoculation), but specific antibodies were not produced (data not shown). On the other hand, *R. sanguineus* infested dog sera recognized rBmCRT (Fig. 6c), while non-infested dog sera did not, suggesting that *R. sanguineus* also secretes a CRT-like protein into the host, producing a humoral response against epitopes that are present in rBmCRT.

4. Discussion

We have isolated and characterized a full coding *B. microplus* cDNA sequence that encodes a 47.7 kDa protein. The deduced sequence of BmCRT shows a high similarity with full-length CRT sequences from other organisms. At least three other lines of evidence suggest that Bmcrt encodes the entire coding sequence: (i) the presence of a putative initiation codon, (ii) a probable signal peptide, and (iii) the isolation of three other independent cDNA clones with same size and sequence at their ends. Furthermore, the predicted size and p*I* of BmCRT lies within the range found for CRTs (Fliegel et al., 1989; Huggins et al., 1995; Khalife et al., 1993; Tsuji et al., 1998). The highly acidic C-domain is the cause of the anomalous migration of CRTs in SDS–PAGE (Coppolino and Dedhar, 1998), and can explain why rBmCRT and BmCRT appears as 55–60 kDa proteins in Western-blots. As expected, BmCRT showed higher sequence conservation with arthropod CRTs, but tick CRTs showed a phylogenetic relation closer to mammalian sequences than to any other invertebrate parasite sequence. Therefore, if CRTs from ticks and other parasites perform similar functions in the relationship with their mammalian hosts, they probably have evolved independently.

Numerous functions have been assigned for mammalian CRTs, including molecular chaperone activity (Svaerke and Houen, 1998), Ca^{+2} cellular buffering and integrin-mediated Ca^{+2} release/influx (Kwon et al., 2000; Mery et al., 1996), C1q-binding (Kishore et al., 1997; Kovacs et al., 1998), endothelial nitric oxide production (Kuwabara et al., 1995). Such multiple and diverse functions make CRT-like proteins of parasites prominent candidates to perform important roles in the relationship with their hosts. Calcium-binding activity was reported for CRTs of *S. mansoni* (Khalife et al., 1994), *S. japonicum* (Huggins et al., 1995), *D. immitis* (Tsuji et al., 1998), and *L. donovani* (Joshi et al., 1996). BmCRT shows a high conservation in the P-domain pointing to the likely presence of a putative high-affinity/low-capacity Ca^{+2}-binding region, and the highly acidic C-domain is consistent with a high-capacity/low-affinity Ca^{+2}-binding activity, which are characteristic features of human CRT (Baksh and Michalak, 1991). Also, *N. americanus* and human CRT have been shown to

bind C1q, and BmCRT shows six human-like C1q-binding sites that have been described to be associated with this activity (Kasper et al., 2001; Kovacs et al., 1998).

CRT was reported as an ER resident protein, presenting a classical retention signal (KDEL) and a signal peptide (Fliegel et al., 1989; Michalak et al., 1992), features that seem to be conserved even in the protist *E. gracilis* (Navazio et al., 1998). But, some parasites present variants of the ER retention signal, such as HDEL, found in schistosomes (Huggins et al., 1995; Khalife et al., 1993), and KEDL, in *L. donovani* (Joshi et al., 1996). BmCRT and the CRTs of *A. americanum* and *N. americanus* present the variant HEEL. Jaworski et al. (1995) suggested that the missing KDEL sequence in the *A. americanum* protein, which is secreted in the saliva, could contribute to its routing into a secretory pathway rather than being retained in the ER. CRT is ubiquitously expressed in mammals, being found in all nucleated cell types (Khanna and Waisman, 1986). In arthropods CRT expression has not been analyzed. In this regard Bmcrt showed to present the same ubiquitous expression found in mammals and to be also secreted in the *B. microplus* saliva. The same findings are observed for the *D. immitis* CRT, which do not possess an ER retention signal, and *N. americanus* CRT (Kasper et al., 2001; Tsuji et al., 1998). In *N. americanus* it was suggested that the ubiquitous expression of CRT presumably reflects its nature as an ER-resident protein (Kasper et al., 2001). As already mentioned, the many functions performed by CRT indicate that the biological role of a tick-secreted form may be linked to the modulation of the host immune system and haemostasis (Jaworski et al., 1995). On the other hand, a CRT present in all tissues would probably be responsible for the same intracellular functions commonly assigned to this protein.

CRTs have, in many situations, been shown to be highly immunogenic to their mammalian hosts. *D. immitis* CRT was recognized by sera of infested dogs (Tsuji et al., 1998). Human patients suffering from onchocerciasis (Rokeach et al., 1994), schistosomiasis (Khalife et al., 1993), infested with *T. cruzi* (Marcelain et al., 2000) or *N. americanus* (Pritchart et al., 1999) showed to present antibodies that recognize the CRT of the respective parasites. *A. americanum* CRT was recognized by sera from rabbits and humans that suffered tick bites (Sanders et al., 1998, 1999). Conversely, BmCRT did not produce a humoral response in *B. microplus*-infested bovines, although rBmCRT was recognized by *R. sanguineus*-infested dog sera. Anti-CRT antibodies cross-reactivity has already been demonstrated between ticks (Sanders et al., 1998, 1999). Taken together these data indicate that CRT secretion in the saliva is a common process among ticks, but its recognition by the host immune system may not be. This is reinforced by the fact that we were not able to produce a hyperimmune serum against rBmCRT by active immunization of a bovine, while the native and recombinant forms seem to be immunogenic to rabbits. Analogously, guinea pigs develop a significant resistance following repeated infestations of *R. sanguineus* while dogs, the natural hosts, do not (Szabó et al., 1995). This fact is explained by the triggering of different immune reactions in response to infestations of natural and unnatural hosts, which were suggested to be derived from specific evasion mechanisms developed by *R. sanguineus* against the immune system of dogs (Szabó et al., 1995).

The possible use of CRT for the development of protective immunity against the parasites *N. americanus* and *Schistosoma* spp. has been suggested (El Gengehi et al., 2000; Khalife et al., 1994; Pritchard et al., 1999). Concerning ticks, rabbits immunized with *A. americanum* CRT exhibited necrotic lesions in the tick bite sites, indicating that the immune reaction could disrupt the feeding cycle (Jaworski et al., 1995). Furthermore, the antibody levels to *A. americanum* CRT increase in humans after exposure to *I. scapularis* and are correlated with tick engorgement indices (Sanders et al., 1999). However, the analysis of rBmCRT as an imunoprophylatic tool faces its low immunogenicity in bovines, which could possibly be overcome using different adjuvants and inoculation strategies or by conjugating rBmCRT to highly immunogenic proteins.

It must also be considered that *B. microplus* is a one-host tick. Other ticks change hosts throughout their lifetime. These two strategies probably counteract similar problems of ectoparasitism and haematophagy differently. The lack of a humoral response against BmCRT in bovines may reflect one of these differences and could possibly indicate some systems that are essential to be evaded in order to allow the *B. microplus* survival in its natural host.

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