cDNA cloning, expression and characterization of a *Boophilus microplus* paramyosin

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SUMMARY

The tick *Boophilus microplus* is a 1-host tick that causes important losses to bovine herds, and protective antigens are being investigated in order to develop vaccines that avoid the use of acaricides. Paramyosins are multi-functional invertebrate muscle proteins, whose roles may include host immunomodulation, and seem to be a prominent candidate in a schistosomiasis vaccine. We report here the cloning, expression and characterization of a *B. microplus* paramyosin (BmPRM). Sequence analysis of the full length coding sequence cDNA shows high identity to other arthropod paramyosin sequences, and the predicted molecular weight, pI and secondary structure are consistent with a typical paramyosin. Western-blot expression analysis indicates the presence of BmPRM in all tissues and developmental stages tested, but not in saliva. The recombinant protein (rBmPRM) was shown to bind both IgG and collagen. Possible implications of these activities with host evasion mechanisms are discussed.

Key words: paramyosin, *Boophilus microplus*, IgG binding protein, collagen binding protein, tick.

INTRODUCTION

Ixodidae ticks are blood-sucking parasites that infest a wide array of mammals. *Boophilus microplus* is a 1-host tick that causes major losses to bovine herds, especially in tropical regions. Major efforts have been expended to develop immunoprophylatic tools in order to achieve better control of the parasite, minimizing or eliminating the use of acaricides that raises costs and contaminates milk, meat and the environment (Nolan, 1985). Possible immunogens have been described (Mulenga, Sugimoto & Onuma, 2000) and vaccines based on the concept of concealed antigens (Willadsen & Kemp, 1988) are being commercialized (Willadsen *et al.* 1995; Rodriguez *et al.* 1995*a*). Unfortunately, the degree of protection achieved is not complete (Willadsen & Kemp, 1988). The use of acaricides is still required in the best scenario, and field trials showed considerable variation among the different regions tested (Rodriguez *et al.* 1995*b*), possibly resulting from polymorphisms of the antigens (Garcia-Garcia *et al.* 2000). The discovery of new protective antigens would be

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helpful in the optimization of current vaccines or in the design of novel ones.

Salivary molecules are well-known modulators of host immune systems and haemostasis, and are considered essential for the maintenance of parasite survival in the tick-mammalian relationship (Ribeiro, 1989, 1995; Wikel, 1999). Blockage of these immunomodulatory molecules appears promising for tick control. However, the analysis of such molecules is confounded by the small amounts of saliva that are available from ticks, which often makes their isolation and further characterization unfeasible. The identification of tick salivary cDNAs and the production of recombinant proteins is a more tractable alternative.

We show in this article the molecular cloning, characterization, expression in a prokaryotic system, and the analyses of some activities of a salivary gland paramyosin of *B. microplus* (BmPRM). Paramyosins are muscle proteins found within a number of different invertebrates, including arthropods, molluscs, annelids, crustaceans, nematodes and echinoderms (Maroto *et al.* 1995). Paramyosin is also found as part of the tegument of *Schistosoma mansoni* (Matsumoto *et al.* 1988; Gobert, 1998), and is one of the most promising candidates as a vaccine against schistosomiasis (Kallina & McMannus, 1997; Gobert, 1998). The possible roles of this protein within

the tick and in the tick–bovine relationship are also discussed.

MATERIALS AND METHODS

Ticks

B. microplus ovipositing 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 by feeding on calves, housed in individual pens on slatted floors.

Harvest of saliva

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 in phosphate-buffered saline, pH 7·2 (PBS; 0·01 M sodium phosphate, 0·15 M NaCl). Saliva was then collected directly from tick mouthparts.

Antigen preparation

Fully and partially engorged female ticks were washed with PBS. The dorsal surface was dissected with a scalpel 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 *et al.* (1994). Protein concentrations of the extracts were measured using the Bradford method (1976) with bovine serum albumin as standard.

Antisera

Antibodies against salivary gland extracts and the purified BmPRM recombinant protein (rBmPRM) were raised in rabbits.

Synthesis and screening of the salivary glands cDNA library

A 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. Poly A$^+$ RNA was obtained using the Micro-Fast Track™ Kit (Invitrogen). Immunological screening was performed using serum from a rabbit immunized with *B. microplus* salivary glands extract, as previously described (Rosa de Lima *et al.* 2002).

DNA sequencing and sequence analysis

DNA sequencing of all cDNAs isolated was performed on an ABI-PRISM 377 automated DNA sequencer (PerkinElmer, Foster City, CA) at the Molecular Genetics Facility, Georgia, USA. The FASTA algorithm (Pearson & Lipman, 1988) was used to analyse the nucleotide and deduce amino-acid sequence homologies with previously reported sequences within databases. Multiple alignment of paramyosin sequences was performed with CLUSTALW (Thompson *et al.* 1994). The locations of coiled-coil regions were predicted by the PAIRCOIL program (Berger *et al.* 1995).

Construction of a plasmid over-expressing rBmPRM

In order to allow the expression and purification of rBmPRM the coding region of the cloned cDNA (named Bmprm) was amplified by PCR and sub-cloned into the pGEX-4T3 vector (Amersham-Pharmacia). The upstream (CCCCCCGAATTC-ATGTCTAGCAGGAGCAGC) and downstream (TTTTCCCCCCGCGGCCGCTTAGAAGTT-CTGGCTGGAC) primers included *Eco*RI and *Not* I restriction sites, respectively, which were used for ligation to the vector. Correct cloning was confirmed by sequencing, and the recombinant plasmid named pGEX-PRM.

Protein expression and purification

BmPRM expression was performed in the BL21 *Escherichia coli* strain. Lysogens of BL21/pGEX-PRM were prepared after growth in Luria-Bertani medium. Recombinant protein expression was induced with IPTG 0·1 mM. Cell pellets from 2000 ml cultures 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 %, and centrifuged for 15 min/12000 ***g***. The protein purification was then performed by use of glutathione-Sepharose 4B (Amersham-Pharmacia) affinity chromatography. The supernatant was loaded on the column that had been equilibrated and washed with buffer A (140 mM NaCl, 2·7 mM KCl, 10 mM Na$_2$HPO$_4$, 1·8 mM KH$_2$PO$_4$), 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 10 % SDS–PAGE (Laemmli, 1970) stained with Coomassie Blue G-250. GST was produced using the same protocol, from lysogens of BL21/pGEX-4T1 (Amersham-Pharmacia), with the exception that thrombin was not added and protein elution was performed directly with buffer B.

Western blots and dot-blot analyses

For Western blot analyses, samples were resuspended in buffer containing 2 % SDS, 250 mM Tris,

pH 6·8, 0·025 % bromophenol blue, 5 % glycerol, 10 % β-mercaptoethanol and 5 M urea, separated by SDS–PAGE 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 sheets were blocked with BLOTTO (5 % cow non-fat dry milk-PBS) for 2 h at room temperature. Anti-rBmPRM serum diluted 1:2000 was incubated overnight at 4 °C. Prior to incubation with the antigens, all sera were diluted in an *E. coli* BL21 strain lysate expressing the pGEX-4T3 vector and incubated for 3 h at room temperature to allow the 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). After 3 washes with BLOTTO, anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Sigma) or peroxidase (Sigma), diluted 1:5000 and 1:2000 in BLOTTO respectively, were incubated for 1 h at room temperature. The membranes were then washed and stained as described for alkaline phosphatase (Rosa de Lima *et al.* 2002) and peroxidase (Da Silva Vaz *et al.* 1998) conjugates. Molecular weight standards were purchased from Bio-Rad (High Range).

Dot-blot analysis was performed using 5 μg of rBmPRM in each spot. Native protein was applied to the nitrocellulose membrane diluted in PBS while denatured protein was applied after 10 min of boiling, diluted in PBS plus SDS 0·5 % and β-mercaptoethanol 5 %. Membranes were blocked with BLOTTO for 2 h at room temperature. Anti-BmPRM serum diluted 1:2000 or non-infested bovine serum diluted 1:20 were incubated for 1·5 h at room temperature. Prior to incubation with the antigens, all sera were diluted in an *E. coli* BL21 strain lysate expressing the pGEX-4T3 vector and incubated for 3 h at room temperature to allow the absorption of anti-*E. coli* and anti-vector derived protein antibodies. Preparation of the *E. coli* BL21 strain lysate was performed according to the method described by Rott *et al.* (2000). After 3 washes with BLOTTO, anti-rabbit IgG or anti-bovine IgG antibodies conjugated to alkaline phosphatase (Sigma) diluted 1:5000 and 1:2000 in BLOTTO, respectively, were incubated for 1 h at room temperature. After 3 washes with PBS and once with development buffer (5 mM MgCl₂, 100 mM NaCl, 100 mM Tris, pH 9·5), membranes were stained with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT).

IgG purification

A pool of bovine sera from non-infested cattle was dialysed with 20 mM sodium phosphate buffer, pH 8·4, and applied to a protein G-Sepharose column (Amersham-Pharmacia) equilibrated with the same buffer and eluted with 100 mM glycine–HCl buffer, pH 2·7. The fractions containing IgG were dialysed against PBS.

ELISA

Microtitration plates were coated with 2·5 μg of BSA (Sigma), rBmPRM or GST per well in 20 mM carbonate buffer (pH 9·6) by overnight incubation at 4 °C in a wet chamber (Harlow & Lane, 1988). They were washed 3 times in BLOTTO and blocked by additionally incubating for 1 h at 37 °C with BLOT-TO. Either purified bovine IgG or total bovine serum were diluted in BLOTTO and incubated for 1 h at 37 °C. Prior to incubation with the antigens, both bovine serum and purified IgG were also added to an *E. coli* lysate (same procedure used for the Western blots) to remove any cross-reacting antibodies to *E. coli* and vector-derived proteins. PBS was also incubated with the *E. coli* lysate and used as a negative control (no antibodies or serum were added). The plates were then washed with BLOTTO 3 times and incubated for 1 h at 37 °C with a secondary antibody conjugated to peroxidase (anti-bovine IgG, Sigma, diluted 1:2000). After 3 washes with PBS, the chromogen and substrate were added (3·4 mg *o*-phenylenediamine, 5 μl of H₂O₂ in 0·1 M citrate-phosphate buffer, pH 5·0) and incubated for 15 min at room temperature in a dark room. The reactions were stopped with 12·5 % H₂SO₄ and the optical densities (OD) were determined at 492 nm.

Collagen binding

Seventeen mg of collagen type I (Sigma) were washed initially with PBS–NaCl 0·65 M (PBS plus NaCl up to 0·65 M) and subsequently with PBS, and then incubated overnight at 4 °C with 300 μg of GST and 300 μg of rBmPRM in PBS. Collagen was then washed successively with PBS, PBS–NaCl 0·2 M (PBS plus NaCl up to 0·2 M), PBS–NaCl 0·25 M (PBS plus NaCl up to 0·25 M) and PBS–NaCl 0·3 M (PBS plus NaCl up to 0·3 M). Elution was performed with PBS–NaCl 0·65 M. All fractions were then concentrated and ionic strength was adjusted to physiological conditions using an Amicon microconcentrator filter (pore exclusion of 30 000).

RESULTS

Isolation and sequence analysis of BmPRM

A single paramyosin-like cDNA clone (BmPRM) was obtained from 9000 cDNA clones by immunological screening from a salivary gland expression library. An antiserum raised in a rabbit against partially engorged salivary glands was used as a probe. The BmPRM sequence encodes a 2922 cDNA fragment with an open reading frame of 873 amino acids having high similarity to full-length paramyosin sequences (Fig. 1). The first initiation codon fulfills the criteria of Kozak for a ribosomal binding site

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Onchocerca       : MSGSLYRSPSAALYKSPSMSAFSGLPAAFGSMSVADLGSLTRLEDKIRLLQEDLESERE : 59
Dirofilaria      : -------------------------AFGSMSVADLGSLTRLEDKIRLLQEDLESERE : 32
Brugia           : MSGSLYRSPSAALYKSPSMSAFGGLPAAFGSMSVADLGSLTRLEDKIRLLQEDLESARE : 59
Sarcoptes        : MSA----RSAKFMYRSG-N---AGASGDLSVEYGTDLGALTRLEDKIRLLSDDLESERE : 51
Dermatophagoides : ---------------------------------------------------------- : -
Boophilus        : MSS----RSSKYMYKS------SGGAGDISIEYGTDLGALTRLEDKIRLLQEDLESERE : 49
Drosophila       : MSSSQAVRSSKYSYRATST---GPGTADVNIEYIQDISSLSRLEDKIRLLQDDLEVERE : 56

Onchocerca       : LRNRIERERADISVQLIALTDRLEDAEGTTDSQIESNRKREAEIQKLRKLLEESQLENE : 118
Dirofilaria      : LRNRIERERADISVQLIALTDRLEDAEGTTDSQIESNRKREAEIQKLRKLLEESQLENE : 91
Brugia           : LRNRIERERADISVQLIALTDRLEDAEGTTDSQIESNRKREAEIQKLRKLLEESQLENE : 118
Sarcoptes        : MRQRIEREKAELQIQVMSLSERLEEAEGSSESVVEMNKKRDSELAKLRKLLEDVHLESE : 110
Dermatophagoides : ----------------------------------MNKKRDSELAKLRKLLEDVHIESE : 24
Boophilus        : LRQRIEREKSDITVQLMQLSDRLEEAEGSSETVVEMNKKRDTELSKLRKLLEDVHLESE : 108
Drosophila       : LRQRIEREKADISVQVIQMSERLEEAEGGAEHQFEANRKRDAEILKLRKLLEDVHLESE : 115

Onchocerca       : DAMNVLRKKHQDACLDYTEQIEQLQKKNSKIDRERQRLQHEVIELTAAIDQLQKDKHLA : 177
Dirofilaria      : DAMNVLRKKHQDACLDYAEQIEQLQKKNSKIDRERQRLQHEVIELTATIDQLQKDKHLA : 150
Brugia           : DAMNILRKKHQDACLDYAEQIEQLQKKNSKIDRERQRLQHEVIELTATIDQLKKDKHLA : 177
Sarcoptes        : ETAHHLRQKHQAAIQEMQDQQLDQVQKAKNKSDKEKQKFQAEVFELLAQLETANKEKLTA : 169
Dermatophagoides : ETAHHLRQKHQAAIQEMQDQQLDQLQKAKNKSDKEKQKFQAEVFELLAQLETANKEKLTA : 83
Boophilus        : ETAHHLRKKHQEAVAEMQEQMDLMTKAKSKAEKERQKFQAEVYELLAQVENTNKEKITI : 167
Drosophila       : ETTLLLKKKHNEIITDFQEQVEILTKNKARAEKDKAKFQTEVYELLSQIESYNKEKIVS : 174

Onchocerca       : EKAAERFEAQTIEISNKVEDLNRHVNDLAQQRQRLQAENNDLLKEIHDQKVQLDNLQHV : 236
Dirofilaria      : EKAAERFEAQTIEISNKVEDLNRHVNDLAQQRQRLQAENNDLLKEIHDQKVQLDNLQHV : 209
Brugia           : EKAAERFEAQTVEISNKVEDLNRHVNDLAQQRQRLQAENNDLLKEIHDQKVQLDNLQHV : 236
Sarcoptes        : MKTVEKLEYTVHEINIKIEEINRTVIELTSQKTRLSQENTELIKEVHEHKMQLDNANHL : 228
Dermatophagoides : LKNVEKLEYTVHEINIKIEEINRTVIELTSHKQRLSQENTELIKEVHEVKLQLDNANHL : 142
Boophilus        : QKTVEKLEHTVYEINIRIEELNRTVTEVTAQRTRLSAENAEYLKEVHELKVSLDNVNHL : 226
Drosophila       : EKHISKLEVSISEINVKIEELNRTVIDISSHRSRLSQENIEITKDVQDLKVQLDTVSFS : 233

Onchocerca       : KYQLAQQLEEARRRLEDAERERSQLQAQLHQVQLELDSVRTALDEESAARAEAEHKLAL : 295
Dirofilaria      : KYQLAQQLEEARRRLEDAERERSQLQAQLHQVQLELDSVRTALDEESAARAEAEHKLAL : 268
Brugia           : KYQLAQQLEEARRRLEDAERERSQLQAQLHQVQLELDSVRTALDEESAARAEAEHKLAL : 295
Sarcoptes        : KQQLAQQLEDTKHRLEEEERKRASLENHAHTLEVELESLKVQLDEESEARLELERQLTK : 287
Dermatophagoides : KTQIAQQLEDTRHRLEEEERKRASLENHAHTLEVELESLKVQLDEESEARLELERQLTK : 201
Boophilus        : KSQLATQLEDTRRRLEDEERKRASLESSMHTLEVELESLKVQLEEESEARLEVERQLVK : 285
Drosophila       : KSQVISQLEDARRRLEDEDRRRSLLESSLHQVEIELDSVRNQLEEESEARIDLERQLVK : 292

Onchocerca       : ANTEITQWKSKFDAEVALHHEEVEDLRKKMLQKQAEYEEQIEIMLQKISQLEKAKSRLQ : 354
Dirofilaria      : ANTEITQWKSKFDAEVALHHEEVEDLRKKMLQKQAEYEEQIEIMLQKISQLEKAKSRLQ : 327
Brugia           : ANTEITQWKSKFDAEVALHHEEVEDLRKKMLQKQAEYEEQIEIMLQKISQLEKAKSRLQ : 354
Sarcoptes        : ANGDAASWKSKYEAELQAHADEVEELRRKMAQKISEYEEQLEALLNKCSSLAKQKSRLQ : 346
Dermatophagoides : ANGDAASWKSKYEAELQAHADEVEELRRKMAQKISEYEEQLEALLNKCSSLEKQKSRLQ : 260
Boophilus        : ANADAAAYKTKYETEVQAHADEVEELRRKMAQKISEYEEQLEALLTRCSNLEKQKSRLQ : 344
Drosophila       : ANADATSWQNKWNSEVAARAEEVEEIRRKYQVRITELEEHIESLIVKVNNLEKMKTRIA : 351

Onchocerca       : SEVEVLIVDLEKAQNTIAILERAKEQLEKTVNELKVRIDELTVELEAAQREARAALAEL : 413
Dirofilaria      : SEVEVLIVDLEKAQNTIAILERAKEQLEKTVNELKVRIDELTVELEAAQREARAALAEL : 386
Brugia           : SEVEVLIVDLEKAQNTIAILERAKEQLEKTVNELKVRIDELTVELEAAQREARAALAEL : 413
Sarcoptes        : SEVEVLIMDLEKATTHAQQLEKRVAQLEKLNLDLKNKLEEVTMLMEQAQKEARAKAAEL : 405
Dermatophagoides : SEVEVLIMDLEKATRHAQQLEKRVAQLEKINLDLKNKLEEVTMLMEQAQKELRVKIAEL : 319
Boophilus        : SEVEVLIMDLEKATAHAQNLEKRVAQLEKLNIDLKSKVEELTILLEQSQRELRQKVAEI : 403
Drosophila       : SEVEVLIIDLEKSNNSCRELTKSVNTLEKHNVELKSRLDETIILYETSQRDLKNKHADL : 410

Onchocerca       : QKMKNLYEKAVEQKEALARENKKLQDDIHEAKEALADANRKLHELDLENARIAGEIREL : 472
Dirofilaria      : QKMKNLYEKAIEQKEALARENKKLQDDIHEAKEALADANRKLHELDLENARIAGEIREL : 445
Brugia           : QKLKNLYEKAVEQKEALARENKKLQDDIHEAKEALADANRKLHELDLENARIAGEIREL : 472
Sarcoptes        : QKLQHEYEKLRDQRDALARENKKLTDDLAECKSQLNDAHRRIHEQEIEIKRLENEREEL : 464
Dermatophagoides : QKLQHEYEKLRDQRDQLARENKKLTDDLEAKSQLNDAHRRIHEQEIEIKRLENERDEL : 378
Boophilus        : QKLQHEYEKMREQRDALQRENKKLVDDLSEAKSQLADAIRRTHEYELEIKRLENERDEL : 462
Drosophila       : VRTVHELDKVKDNNNQLTRENKKLGDDIHEAKGAINELNRRLHELELELRRIENERDEI : 469
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Fig. 1. For legend see facing page.

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Onchocerca      : QTALKESEAARRDAENRAQRALAEIQQLRIEMERRIQEKEEEMEALRKNMQFEIDRLTA : 531
Dirofilaria     : QTALKESEAARRDAENRAQRALAEIQQLRIEMERRIQEKEEEMEALRKNMQFEIDRLTA : 504
Brugia          : QTALKESEAARRDAENRAQRALAEIQQLRIEMERRIQEKEEEMEALRKNMQFEIDRLTA : 531
Sarcoptes       : SAAYKEAETIRKQEEAKNQRLTAEIAQVRHDYEKRIAQKEEEIEALRKQYQIEIEQLNM : 523
Dermatophagoides: SAAYKEAETIRKQEEAKNQRLIAEIAQVRHDYEKRIAQKDEEIEALRKQYQIEIEQLNM : 437
Boophilus       : AAAYKEAETIRKQEEAKQRLTAEIAQVRHEYERRIQIKEEEIEALRKQYQIEVEQLNM : 521
Drosophila      : TAAYKEAEAGRKAEEQRGQRLAADFNQYRHDAERRIAEKDEEIEAIRKQTSIEIEQLNA : 528

Onchocerca      : ALADAEARMKAEIARLKKKYQAEIAELEMTVDNLNRANIEAQKTIKKQSEQLKVLQASL : 590
Dirofilaria     : ALADAEARMKAEISRLKKKYQAEIAELEMTVDNLNRANIEAQKTIKKQSEQLKILQASL : 563
Brugia          : ALADAEARMKAEISRLKKKYQAEIAELEMTVDNLNRANIEAQKTIKKQSEQLKILQASL : 590
Sarcoptes       : RLAEAEAKLKTEIARLKKKYQAQITELELSLDAANKANIDLQKTIKKQAIQITELQAHY : 582
Dermatophagoides: RLAEAEAKLKTEIARLKKKYQAQITELELSLDAANKANIDLQKTIKKQAIQITELQAHY : 496
Boophilus       : RLAEAEAKLKTEIARIKKKYQAQITELEMSLDAANKQNMDLQKIIKKQAIQITELQAHY : 580
Drosophila      : RVIEAETRLKTEVTRIKKKLQIQITELEMSLDVANKTNIDLQKVIKKQSIQLTELQAHY : 587

Onchocerca      : EDTQRQLQQTLDQYALAQRKVSAISAELEECKVAIDNAIRARKQAEIDLEEANGRITDL : 649
Dirofilaria     : EDTQRQLQQTIDQYALAQRKVSAISAELEECKVAIDNAIRARKQAEIDLEEANARITDL : 622
Brugia          : EDTQRQLQQTLDQYALAQRKVSAISAELEECKVAIDNAIRARKQAEIDLEEANGRITDL : 649
Sarcoptes       : DEVHRQLQQAVDQLGVTQRRCQAIQAELEEQRIALEQANRAKRQAEQLHEEAVARVNEL : 641
Dermatophagoides: DEVHRQLQQAVDQLGVTQRRCQAIQAELEEMRIALEQANRAKRQAEQLHEEAVVRVNEL : 555
Boophilus       : DEVHRQLQQCADQLAISQRRCQIQAELDEQRVALESALRSKRAAEQSLEESQARVNEL : 639
Drosophila      : EDVQRQLQATLDQYAVAQRRLAGINGELEEVRSHLDSANRAKRTVELQYEEAASRINEL : 646

Onchocerca      : VSINNNITAIKNKLETELSTAQADLDEATKELHAADERANRALADAARAVEQLHEEQEH : 708
Dirofilaria     : VSINNNITAIKNKLETELSTAQADLDEATKELHAADERANRALADAARAVEQLHEEQEH : 681
Brugia          : VSVNNNITAIKNKLETELSTAQADLDEATKELHAADERANRALADAARAVEQLHEEQEH : 708
Sarcoptes       : TTINVNIASAKSKLESEFAALQNDYDEVHKELRISDERVQKLTIELKSTKDLLVEEQER : 700
Dermatophagoides: TTINVNIASAKSKLESEFSALQADYDEVHKELRISDERVQKLTIELKSTKDLLIEEQER : 614
Boophilus       : TTINVNIAAAKNKLESELSALQADYDELHKELRVVDERCQRTIVELKSTKDILVEEQER : 698
Drosophila      : TTANVSLVSIKSKLEQELSVVASDYEEVSKELRISDERYQKVQVELKHVVEQVHEEQER : 705

Onchocerca      : SMKIDALRKSLEEQVKQLQVQIQEAEAAAILGGKRVIAKLETRIRDLETAIDEETRRHK : 767
Dirofilaria     : SMKIDALRKSLEEQVKQLQVQIQEAEAAAILGGKRVIAKLETRIRDLETAIDEETRRHK : 740
Brugia          : SMKIDALRKSLEEQVKQLQVQFRKLKRR-LGGKRVIAKLETRIRDLETAIDEETRRHK : 766
Sarcoptes       : LVKMETVKKSLEQEVRTLHVRIEEVEANAIAGGKRVIAKLESRIRDVEIEVEEERRRHA : 759
Dermatophagoides: LVKLETVKKSLEQEVRTLHVRIEEVEANAIAGGKRVIAKLESRIRDVEIEVEEERRRHA : 673
Boophilus       : YIKVESIKKSLEVEVRNLQVRLEEVEANAIAGGKRVIAKLEARIRDVEIETEEEKKRHA : 757
Drosophila      : IVKLETIKKSLEVEVKNLSIRLEEVELNAVAGSKRIISKLEARIRDLELEIEEEKRRHA : 764

Onchocerca      : ETQGALRKKDRRIKEVQMQVDEEHKMFVMAQDTADRLLEKLNIQKRQIGE-AESLTMAN : 825
Dirofilaria     : ETQGALRKKDRRIKEVQMQVDEEHKMFVMAQDTADRLLEKLNIQKWQIGE-AESLTMAN : 798
Brugia          : ETQGALRKKDRRIKEVQMQVDEEHKMFVMAQDTADRLLEKLNIQKRQIGE-AESLTMAN : 824
Sarcoptes       : ETEKMLRKKDHRLKELLVQNEEDHKQIQLLQEMVDKMNEKVKVYKRQMQETREGMSQQN : 818
Dermatophagoides: ETDKMLRKKDHRVKELLLQ-------------------------------------- : 692
Boophilus       : ETQKILRKKDHRAKELLLQTEEDHKTITMLNDAVEKLNEKVKVYKRQINE-QEGLSQQN : 815
Drosophila      : ETIKILRKKERTVKEVLVQCEEDQKNLILLQDALDKSTAKINIYRRQISE-QEGVSQQT : 822

Onchocerca      : LQRVRRYQRELEDAEGRADQAESSLHLIRAKHRSSVTAGKNAS-ASKIYVLEDDQ---- : 879
Dirofilaria     : LQRVRRYQRELEDAEGRADQAESSLHLIRAKHRSSVVTGKNAS-ASKIYVL-------- : 848
Brugia          : LR-VRRYQRELEDAEGRADQAESSLHLIRAKHRHQLLRAKMLQ-RQKFTFSKMSNRDN- : 880
Sarcoptes       : LTRVRRFQRELEAAEDRADQAESNLSFIRAKHRSWVTTSQVPGGTRQVFVTQEEQSNY- : 876
Dermatophagoides: ----------------------------------------------------------- : -
Boophilus       : LTRVRRFQRELEAAEDRADSAESNLSLIRAKHRSWVTTSQVPGGTRQVFVTEESSQNF- : 873
Drosophila      : TTRVRRFQRELEAAEDRADTAESSLNIIRAKHRTFVTTSTVPG--SQVYIQETTRTITE : 879
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Fig. 1. Multiple alignment of the deduced amino acid sequence of BmPRM and other paramyosin sequences. Genbank accession numbers: *Onchocerca volvulus*, Q02171; *Dirofilaria immitis*, P13392; *Brugia malayi*, Q01202; *Sarcoptes scabiei*, Q9BMM8; *Dermatophagoides farinae*, AAK39511; *Boophilus microplus* (BmPRM), AF479582; *Drosophila melanogaster*, P35415. Dark shading shows identity and light shading shows residues conserved in 5/6 sequences.

(Kozak, 1991). A 36 base pair (bp) 5′ and a 264 bp 3′ untranslated sequence are present, with a putative polyadenylationsignalpositioned24 bpfromthepoly-A tail at the 3′ end. The predicted molecular mass and pI for BmPRM are 102 kDa and 5·53, respectively. Three putative *N*-glycosylation sites are present at residues 189–192, 815–818 and 839–842, and 2 leucine zipper motifs are present at residues
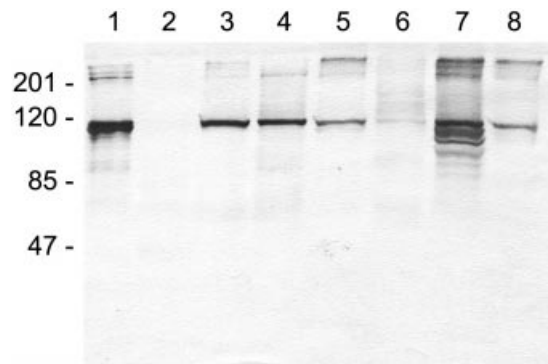
Fig. 2. Western blot analysis of *Boophilus microplus* tissues, saliva and purified rBmPRM. Anti-rBmPRM rabbit serum diluted 1:2000. Lane 1, purified rBmPRM (1 μg); lane 2, saliva from partially engorged females (6 μg); lane 3, partially engorged female salivary glands extract (36 μg); lane 4, fully engorged female fat body extract (20 μg); lane 5, partially engorged female gut extract (18 μg); lane 6, larval extract (96 μg); lane 7, non-engorged total adult female extract (14 μg); lane 8, total adult male extract (49 μg). Molecular weight standards, kDa.
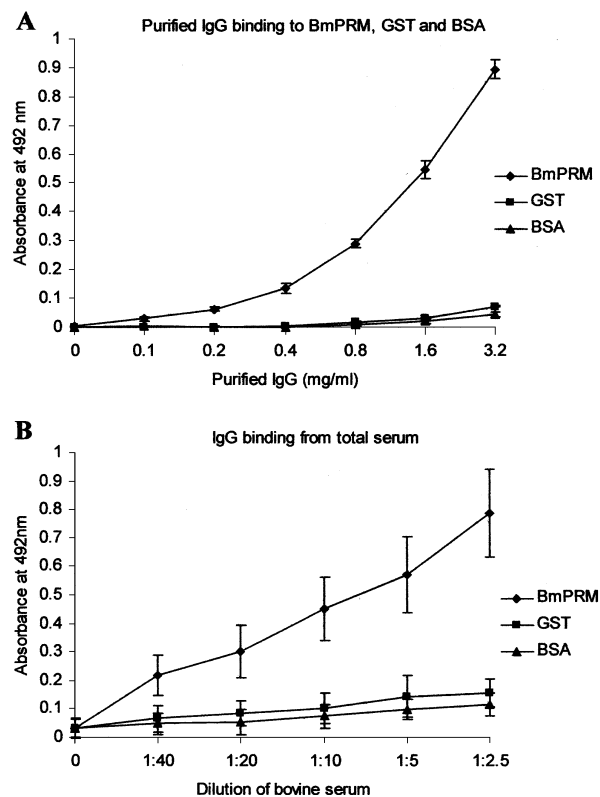

Fig. 3. IgG binding to rBmPRM detected by ELISA. Microplate wells were coated with 2·5 μg of rBmPRM, GST and BSA and incubated with doubling dilutions of purified IgG (from 0·1 to 3·2 mg/ml in (A) or bovine sera (from 1:2·5 to 1:40 in (B)). (A) Data are expressed as means ± S.D. of triplicate experiments; (B) data are expressed as means ± S.D. of 3 separate experiments, each one performed in duplicate, using different non-infested bovine sera. All sera and purified IgG were pre-absorbed against *E. coli* extract prior to incubation with the proteins.

329–350 and 364–385. Several putative phosphorylation sites can also be deduced within the amino acid sequence of BmPRM. A coiled-coil-like secondary structure is predicted for most of the BmPRM sequence, with the exceptions covering the first 26 and the last 27 amino acids. The sequence data have been deposited in GenBank accession number AF479582.

Sequence alignment of BmPRM and 6 other paramyosin sequences shows that BmPRM shares the high conservation observed among paramyosins, especially within arthropods (Fig. 1). BmPRM shares 78%, 62% and 60% identity with *Sarcoptes scabiei*, *Dermatophagoides farinae* and *Drosophila melanogaster*, respectively (data not shown). The identities shared with the parasite nematodes *Onchocerca volvulus*, *Dirofilaria immitis* and *Brugia malayi* are less, being 52%, 52% and 51%, respectively (data not shown), but are representative of the evolutionary conservation of paramyosins even among phylogenetically distant species.

Subcloning and recombinant protein expression of BmPRM

The full coding sequence of BmPRM was amplified by PCR and subcloned into the pGEX-4T3 vector (Amersham-Pharmacia), and then expressed in *E. coli*. rBmPRM, cleaved out from the fusion protein, was used to raise a hyperimmune serum on a rabbit. A high titre hyperimmune serum was also obtained when a bovine was immunized (data not shown).

Identification of BmPRM within tick tissues and developmental stages

The anti-rBmPRM serum was used in Western blot analysis of tick tissues/developmental stages (Fig. 2). The predicted band corresponding to BmPRM (99–105 kDa) was present in all samples tested, with the exception of saliva. A 10-fold greater quantity of saliva was also tested, but the same result was obtained (data not shown). The presence of BmPRM in all tick tissues and developmental stages indicates ubiquitous expression of the BmPRM gene in *B. microplus* whereas the absence of BmPRM in saliva suggests that it is not secreted into the host while feeding. No bands were developed using pre-immune serum (data not shown).

Binding of bovine IgG to rBmPRM

Binding of rBmPRM to bovine IgG was demonstrated by ELISA. rBmPRM, GST (from *Schistosoma japonicum*, used as a fusion protein in the rBmPRM expression; see Materials and Methods section) and BSA were coated onto microplate wells and tested for binding to purified bovine IgG (Fig.
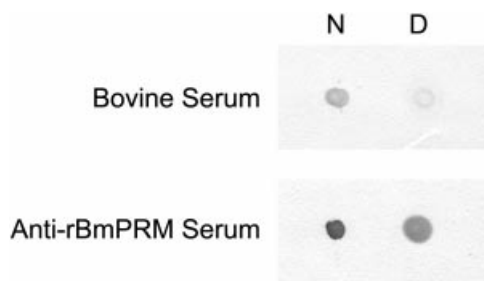
Fig. 4. Dot-blot analysis of IgG binding activity of rBmPRM, (N) before and (D) after denaturation. The same amount of rBmPRM was applied to nitrocellulose membranes and incubated with non-infested bovine serum diluted 1:20. As a control a replicate was incubated with anti-rBmPRM rabbit serum diluted 1: 2000. Both sera were pre-absorbed against *E. coli* extract prior to incubation with the membranes.
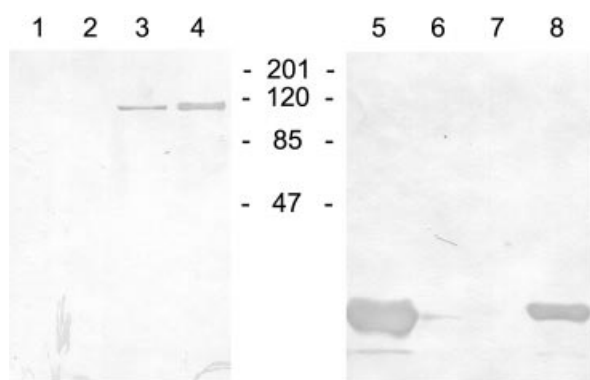

Fig. 5. rBmPRM binds collagen. rBmPRM and GST were incubated with type I collagen and fractions collected following stepwise salt washes. Anti-rBmPRM rabbit serum diluted 1:2000 was used as probe in lanes 1–4 and anti-GST monoclonal antibody diluted 1:3000 in lanes 5–8. Lanes 1 and 5, unbound protein; lanes 2 and 6, last wash fraction (0·3 M NaCl); lanes 3 and 7, eluted protein; lanes 4 and 8, purified rBmPRM (4 μg) and GST (4 μg). Molecular weight standards, kDa.

3A). rBmPRM showed dose-dependent binding over a 0·1–3·2 mg/ml concentration range, whereas GST and BSA did not show significant binding. In order to test if rBmPRM could also bind IgG of whole serum, we tested 3 different bovine sera (from non-infested animals) at 5 dilutions (Fig. 3B). Essentially the same results were obtained when whole sera were used. A dot-blot analysis was performed to compare the relative IgG-binding activity of native and denatured rBmPRM (Fig. 4). The IgG-binding activity was dramatically reduced using the de-natured form, suggesting that binding is dependent on conformation.

rBmPRM binding to collagen

Collagen affinity of rBmPRM was tested by incubating the protein with type I collagen under physio-logical ionic strength conditions, followed by step-wise washes with increasing salt concentrations (up to 0·3 M NaCl). Elution was achieved with a 0·65 M NaCl solution. GST was co-incubated with rBmPRM as a negative control. Fractions were concentrated, separated by SDS–PAGE and ana-lysed by Western blot (Fig. 5). Detection of rBmPRM exclusively in the eluted fraction indicates binding to collagen and that most of the recombinant protein produced retains the binding capacity.

DISCUSSION

In this report we show the isolation, characterization and expression of a full length *B. microplus* cDNA sequence that encodes a 102 kDa protein. The deduced sequence of BmPRM shows high similarity with full length paramyosin sequences from other organisms. Sequence and structural analyses indicate that BmPRM represents a paramyosin, a protein that has been described in several invertebrates (Kagawa *et al.* 1989; Maroto *et al.* 1995; Tsai *et al.* 1998; Mattson, Ljunggren & Bergstrom, 2001). The predicted molecular weight of BmPRM lies within the values found for most paramyosins (Maroto *et al.* 1995). The predicted coiled-coil-like secondary structure is consistent with a rod-like shape, which is characteristic of paramyosins and myosin heavy chains (McLachlan & Karn, 1982; Kagawa *et al.* 1989). Multiple alignment reveals a high degree of conservation between paramyosins and BmPRM. Furthermore, no gaps are present within the predict-ed α-helical coiled-coil region (Fig. 1), suggesting that this portion is essential to protein function. This is demonstrated by the fact that point mutations in the *Caenorhabditis elegans* paramyosin within this region caused alterations in muscle structure and function (Gengyo-Ando & Kagawa, 1991).

Paramyosin is found in the musculature of a large number of invertebrates (Maroto *et al.* 1995). It is also found in the tegument of *S. mansoni* (Matsumoto *et al.* 1988; Gobert, 1998), extending the possible functions of this protein. Exposure of paramyosin to the host offers the potential for paramyosin-based vaccines (Kalinna & McManus, 1997). Also, muscle proteins were described as protective immunogens against the stable fly *Stomoxys calcitrans* (Schlein & Lewis, 1976), which indicates that direct damage to muscle derived from an immunological response may occur. Differential tissue expression of para-myosin has been studied in *D. melanogaster* (Maroto *et al.* 1995; Arredondo *et al.* 2001). To our knowledge, other studies on the distribution of paramyosin in tissues and different development stages of arthropods have not been reported. Ex-pression analysis of BmPRM clearly indicates that tick organs with no prominent musculature, like the fat body and salivary glands, give a strong signal

for paramyosin. The apparent absence of BmPRM in the saliva is not unexpected, since BmPRM does not possess a signal sequence.

Paramyosin of *Taenia crassiceps* (Kalinna & McManus, 1993), *S. japonicum* and *S. mansoni* (Loukas *et al.* 2001) have been shown to bind IgG non-immunologically. Schistosomes mask their surface with host proteins, including immunoglobulins (Tarleton & Kemp, 1981); paramyosin is probably involved in this mechanism by acting as an Fc receptor on the parasites' surface (Loukas *et al.* 2001). This role of surface paramyosin partially explains the origin of host protection following immunization with the protein, as specific anti-paramyosin antiserum interferes with IgG binding both *in vitro* and in the tegument of living schistosome parasites (Loukas *et al.* 2001). We have demonstrated comparable activity with rBmPRM, which binds both purified IgG and IgG present in intact serum, and is dependent on protein conformation. However, further studies are needed to determine whether BmPRM acts as an Fc receptor. If BmPRM does not make contact with the host immune system, it cannot be a potential target for vaccine development. However, tick immunoglobulin binding proteins have been described (Wang & Nuttall, 1994, 1995), and a mechanism of selective IgG removal and excretion via salivation has been proposed (Wang & Nuttall, 1995). In this model, specific proteins would be responsible for sequestering and transporting IgG to the salivary glands, via haemolymph. As discussed by Wang & Nuttall (1999), such a mechanism could represent a self-defence system. IgGs would be delivered back into the host, possibly competing for the Fc receptors of cells like mast cells and basophils, which are thought to be essential for tick rejection by the host (Brown & Askenase, 1985; Worms, Askenase & Brown, 1988; Wang & Nuttall, 1999). BmPRM did not show significant similarity to tick immunoglobulin binding proteins when compared within sequence databases (data not shown). Whether BmPRM functions as a physiological defence mechanism remains to be determined.

Another characteristic of native and recombinant paramyosins of many species is its ability to bind collagen (Laclette *et al.* 1990, 1992; Landa *et al.* 1993). This activity is especially important considering that paramyosin inhibits the classical pathway of the complement system, possibly by binding to the collagen-like stalks of Clq (Laclette *et al.* 1992). rBmPRM binds collagen, which suggests that BmPRM may also be a complement inhibitor. In addition, the presence of BmPRM in the gut may indicate its possible exposure to the bloodmeal, and consequently to the complement system. Specific uptake of immunoglobulins from the gut into the tick *Amblyomma americanum* haemolymph has been described, and the probable involvement of IgG binding proteins in the process has been suggested (Jasinska, Jaworski & Barbour, 2000). BmPRM could, in this situation, perform two distinct functions related to host immune system evasion.

The potential of paramyosin as a vaccine candidate against schistosomiasis has been demonstrated (Pearce *et al.* 1988; McManus *et al.* 1998, 2002; Chen *et al.* 2000; Zhou *et al.* 2000), and the IgG and collagen binding activities described in the paramyosins of worms and molluscs (Laclette *et al.* 1992; Loukas *et al.* 2001), which are the likely ultimate targets of the immunological attack, were shown to be conserved in arthropods, or at least in rBmPRM. The use of immunoglobulin binding proteins as immunogens in anti-tick vaccines has been proposed, and an experiment using a recombinant IgG binding protein in guinea-pigs resulted in a prolongation of the engorgement time required by female *Rhipicephalus appendiculatus* (Wang & Nuttall, 1999). Although BmPRM is not apparently in contact with the host immune system, its presence in several organs and developmental stages of the tick deserves greater attention. It may reflect the importance of BmPRM in functions related to host immune system evasion and, as such, provide a target for immunoprophylatic intervention, as a new 'concealed' antigen.

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