A novel neutralization sensitive and subdominant RAP-1-related antigen (RRA) is expressed by *Babesia bovis* merozoites

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SUMMARY

Objective. The *Babesia bovis* genome encodes a *rap-1* related gene denominated RAP-1 related antigen (RRA). In this study, we analysed the pattern of expression, immunogenicity and functional relevance of RRA. **Methods.** Phylogenetic analysis was performed using the program Phylip. Expression of *rra* was analysed by Northern blots, RT-PCR, immunoprecipitation, Western blots and immunofluorescence. RRA antigenicity was tested by T-cell proliferation and Western blot analysis, and functional relevance was determined in an *in vitro* neutralization assay. **Results.** RRA is more closely related to RAP-1b of *Babesia bigemina* than to *B. bovis* RAP-1, and it is highly conserved among distinct strains. Transcriptional analysis suggests lower numbers of *rra* transcripts compared to *rap-1*. Immunoprecipitation of metabolically labelled *B. bovis* proteins with antibodies against synthetic peptides representing predicted antigenic regions of RRA confirmed the expression of a ∼43 kDa RRA in cultured merozoites. Antibodies present in *B. bovis* hyperimmune sera, but not in field-infected cattle sera, reacted weakly with recombinant RRA, and no significant stimulation was obtained using recombinant RRA as antigen in T-cell proliferation assays, indicating that RRA is a subdominant antigen. Antibodies against RRA synthetic peptides reacted with merozoites using immunofluorescence, and were able to significantly inhibit erythrocyte invasion in *in vitro* neutralization tests, suggesting functional relevance for parasite survival. **Conclusion.** *B. bovis* express a novel subdominant RAP-1-like molecule that may contribute to erythrocyte invasion and/or egression by the parasite.

Key words: Apicomplexan, *Babesia bovis*, rhoptry-associated protein-1, RAP-1.

INTRODUCTION

Babesia bovis remains a significant health and economic problem for the cattle industry, causing infections with significant mortality and morbidity rates in semi-tropical and tropical regions worldwide (Bock *et al*. 2004). Despite important research efforts in recent years focused on the development of improved methods of control, vaccination with live attenuated parasites is still the most effective method of prevention (Brown and Palmer, 1999; Bock *et al*. 2004; deWaal and Combrink, 2006). Nevertheless, numerous *B. bovis* antigens with potential for development of recombinant subunit vaccines have been identified and some tested in vaccine trials, including the rhoptry associated protein-1 (RAP-1) (Norimine *et al*. 2003). Although RAP-1 is a component in a partially protective multi-antigen vaccine (Wright *et al*. 1992), neither full size nor truncated recombinant RAP-1 induced protection against challenge with virulent *B. bovis*, despite eliciting strong T-cell and B-cell immune responses in cattle vaccination trials (Norimine *et al*. 2003).

The *rap-1* gene family of *Babesia* occurs in all babesial species examined to date (Goff *et al*. 1988; Suarez *et al*. 1991*a*; Dalrymple *et al*. 1993; Ikadai *et al*. 1999; Skuce *et al*. 1996; Kappmeyer *et al*. 1999; Zhou *et al*. 2007). All members of this family have well-defined molecular features such as the presence of a signal peptide, strict conservation of 4 cysteine residues, a 14 amino-acid motif and several other short motifs present in the first 300 amino acids of the molecules (Suarez *et al*. 1991*a*,*b*; 1994; Dalrymple

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et al. 1996). In *B. bovis*, only 2 identical *rap-1* genes were initially identified (Dalrymple *et al.* 1993; Suarez *et al.* 1998). However, in *B. bigemina* the *rap-1* gene family is present as a complex tandem array of 3 different types of *rap-1a, rap-1b* and *rap-1c* genes (Suarez *et al.* 2003). In this parasite, the 3 types of *rap-1* genes are transcribed in merozoites, but only the expression of RAP-1a proteins was demonstrated, suggesting the existence of mechanisms that tightly regulate the expression of *rap-1* genes. Interestingly, genome sequencing revealed that genes encoding the characteristic RAP-1 motifs are also present in the genome of the related apicomplexan parasites *Theileria parva* and *T. annulata* (Pain *et al.* 2005; Gardner *et al.* 2005). However, except for the highly related *Babesia* and *Theileria* intra-erythrocytic parasites, comprehensive or Blast-assisted database searches have failed to demonstrate conservation of the typical RAP-1 motifs in other available microbial or eukaryotic genomes to date.

Typically, RAP-1 molecules are highly immunogenic and *Babesia*-infected animals normally mount high levels of anti-RAP-1 antibodies, a feature that was utilized for the development of several RAP-1-based methods for serological diagnosis (Boonchit *et al.* 2002, 2004; Goff *et al.* 2006; Zhou *et al.* 2007). Yet, despite extensive molecular and immunological characterization, the functional role of the members of the *Babesia rap-1* gene family remains mostly undefined. It was shown that RAP-1 proteins are expressed at least in the sporozoite and merozoite stages of *B. bovis* (Mosqueda *et al.* 2002). In addition, consistent with their rhoptry localization (Sam-Yellowe, 1996), experimental evidence suggests that RAP-1 proteins are involved in erythrocyte invasion. This is supported by the observations that *B. bovis* RAP-1 is able to bind the surface of erythrocytes (Yokoyama *et al.* 2002), and antibodies against RAP-1 are able to partially block invasion of bovine erythrocytes by merozoites and sporozoites (Yokoyama *et al.* 2002; Mosqueda *et al.* 2002).

Taking into account the limitations previously shown by recombinant versions of RAP-1 as vaccine candidates and partial neutralization data, together with its limited capacity for sequence variation (Suarez *et al.* 1998), suggests that expression of RAP-1-related molecules is required for *B. bovis* survival in the face of the strong anti-RAP-1 immune responses known to occur in vaccinated or chronically infected cattle. Thus, we hypothesize that redundant and poorly immunogenic molecules containing the RAP-1 strictly conserved motifs could function as equivalents of RAP-1, thus providing alternative ways to contribute to erythrocyte invasion and/or egression by *B. bovis*, and overall to the survival of the parasite during infection.

In this report we describe the molecular and antigenic characterization of a novel RAP-1-related antigen (RRA) encoded in the genome of *B. bovis*

(Brayton *et al.* 2007). RRA contains all the signature motifs found in all members of the *rap-1* gene family of *Babesia*; it is encoded by a single copy gene, is highly conserved among geographical isolates of *B. bovis*, contains neutralization sensitive epitopes, and is expressed in *B. bovis* merozoites both *in vitro* and during infection in cattle.

MATERIALS AND METHODS

Parasites

The Mo7 biological clone of *B. bovis* was derived by limiting dilution of the Mexico strain as described elsewhere (Rodriguez *et al.* 1983; Hines *et al.* 1989) and maintained as a cryopreserved stabilate in liquid nitrogen (Palmer *et al.* 1982). The Argentina strains R1A and S2P (Anziani *et al.* 1993) (kindly provided by Ignacio Echaide, INTA-Rafaela) and the Texas *B. bovis* strain T2Bo have been previously described (Goff *et al.* 1988). The Mexican strain of *B. bovis* used for infecting steer C97 was described previously (Brown *et al.* 1992). Parasites were grown in long-term microaerophilus stationary-phase culture by previously described techniques (Levy and Ristic, 1980; Hines *et al.* 1989).

DNA and RNA analysis, cloning and sequencing

Genomic DNA was extracted from cultured merozoites by the standard phenol-chloroform procedure. Sequencing was performed with a Prism Ready Reaction DyeDeoxy Terminator cycle sequencing kit and read with an ABI PRISM 373 genetic analyzer (Applied Biosystems). Protein secondary structure analysis and B cell epitope prediction were performed using the BCM Search Launcher: Protein Secondary Structure Prediction available at http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html. Phylogenetic analysis was performed using the program Phylip available at the following website: http://www.genebee.msu.su/services/phtree_reduced.html. Total RNA was extracted with TRiZol (Invitrogen) following the manufacturer's instructions. RT-PCR was performed using total RNA as described previously (Suarez *et al.* 2003), and RRA mRNA was amplified using primers *RR-f* and *RR-r* described below.

Synthetic peptides, expression of proteins, and production and detection of antibodies

The open reading frame (ORF) of *rra* was amplified from DNA extracted from the Mo7 strain by PCR using primers *RR-f* (5-atg aca aat tgt tat ttc atg-3′) and *RR-r* (5′-tat att gtt tat gtt tga tgc-3′). PCR-amplicons were cloned into the vector pBAD/Thio-Topo (Invitrogen) for expression of recombinant RRA (rRRA). Primers *RR-f* and *RR-r* were designed

to allow in-frame cloning of the inserts into the vector to produce expressed thioredoxin fusion proteins. Inclusion bodies from bacteria induced with 0·2% arabinose were prepared by sonication and high-speed centrifugation and dissolved in 6 M urea, 0·15 M NaCl, 0·1 M Tris-HCl, pH 8·0. Solubilized protein was purified by affinity chromatography on Ni^{++} columns. Relative antigen purity was confirmed in Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. It must be noted that the predicted size of the recombinant RRA, as shown in Fig. 4A ($\sim$56 kDa) is increased $\sim$15 kDa when compared to the predicted size of the native protein ($\sim$41 kDa) due to the presence of a $\sim$15 kDa thioredoxin domain at the N-terminal end and a HIS-tag at the C-terminal end of the recombinant protein. The synthetic peptide sRRA-1: RHM EGK RFR SKS KSI TLR RQQ SES YGW DKM KGN KKA KE represents a combination of 3 predicted B-cell epitopes of RRA encompassed between amino acids 146–159; 300–312; and 322–336 of the putative *rra* orf. The synthetic peptide sRRA-1 was coupled to KLH via glutaraldehyde, and used for rabbit immunization, performed by Affinity BioReagents. Briefly, 1 rabbit was inoculated by the intramuscular route with 250 μg of conjugated KLH-peptide mixed in an equal volume of complete Freund's adjuvant. Two further immunizations were similarly performed every 2 weeks with KLH-peptide mixed in incomplete Freund's adjuvant. The anti-sRRA-1 rabbit serum from the last bleeding (collected 3 weeks after the last immunization) was used in the immunoprecipitation, immunofluorescence and neutralization assays. Metabolic labelling of *B. bovis* merozoites in *in vitro* cultures with [^{35}S]-methionine followed by immunoprecipitations, Western-blot analysis, and fixed immunofluorescence was performed as described previously (Johnson *et al.* 1997; Suarez *et al.* 2003; Goff *et al.* 1988 respectively). To minimize non-specific reactivity with *E. coli* antigen present in the rRRA protein in Western blot analysis, serum samples were incubated for 2 h at 37 °C with 50 μg/ml of a lysate of *E. coli* TOP10 cells transformed with the pBAD/TOPO® ThioFusion™ vector (previously ligated with the *B. bovis* non-related gene, Am780 from *Anaplasma marginale* (Brayton *et al.* 2005)). The purified rAm780 protein was used as an irrelevant recombinant protein control in the Western blot analysis shown in Fig. 4A.

Babesia bovis-*infected cattle*

Cow C97 (Brahman×Angus cross) was infected with the Mexico strain of *B. bovis* (Brown *et al.* 1991) and, upon the onset of clinical signs of acute babesiosis, was treated with 3·0 mg of berenil (diaminazine aceturate: Sigma)/kg of body weight as previously described (Brown *et al.* 1991). For immunoprecipitation, serum from a *B. bovis* T2Bo-hyperimmunized steer was used, as previously described (Johnson *et al.* 1997). Serum samples from 3 field *B. bovis*-infected cattle in Argentina as diagnosed by immunonofluorescence, were kindly provided by Dr Ignacio Echaide.

T-cell proliferation assay

A short-term T-cell line (CL) was generated by stimulating peripheral blood mononuclear cells (PBMC) derived from a *B. bovis*-infected animal (C97) with *B. bovis*-membrane and organelle-enriched pellet (CM) for 1 week and resting for 1 week without antigen in the presence of irradiated autologous PBMC as described (Norimine *et al.* 2002). For proliferation assays, 3×10^4 T-cells and 2×10^5 irradiated autologous PBMC were cultured with antigen in a total volume of 100 μl of complete RPMI 1640 medium in triplicate wells of round-bottom 96-well plates at 37 °C in a 5% CO_2-humidified atmosphere. Antigens used in the proliferation assays were CM, recombinant RAP-1, recombinant RRA, and control recombinant protein MSP-5 (*A. marginale*). All recombinant proteins were produced and purified with the same methodology described for RRA. To measure proliferation, cells were incubated for the final 18 h of culture with 0·25 μCi of [^{3}H]thymidine (Dupont, New England Nuclear, Boston, MA, USA), after which, radio-isotope labelled nucleic acids were harvested onto glass filters and counted in a beta counter.

In vitro *neutralization test*

Inhibition of *B. bovis* merozoites was performed as described elsewhere (Suarez *et al.* 2000). Briefly, 5×10^5 Mo7 *B. bovis* merozoites were incubated with pre-immune or anti-sRRA-1 rabbit immune sera, diluted 1:5 in culture medium, for 30 min at 4 °C. An equal volume of 5% (v/v) of bovine erythrocytes in culture medium was added prior to incubation in triplicate wells of 96-well plates at 37 °C in a 5% CO_2 atmosphere. The percentage of parasitized erythrocytes (PPE) was determined after 24, 48 and 72 h by microscopical examination of 2000 erythrocytes in Giemsa-stained smears prepared from each well. Results from 3 replicates were analysed by one-tailed Student's *t*-test with a *P* value of <0·05 and a Bonferroni correction for multiple comparisons.

RESULTS

The Babesia bovis *T2Bo genome sequence contains a previously uncharacterized RAP-1- related gene*

Blast searches of the T2Bo *B. bovis* genome with the *B. bovis* RAP-1 predicted amino acid sequence

resulted in highly statistically significant identity (Blast E value: $2e^{-20}$) with the predicted ORF of a previously unknown gene (NCBI Reference Sequence: XP_001610950.1). Virtual translation of the protein encoded by this gene, here termed RAP-1-related antigen (RRA), revealed an ORF of 349 amino acids, predictive of a 41·5 kDa protein containing a signal peptide of 22 amino acids. The RRA predicted amino acid sequence shows 30% identity and 55% overall homology when compared to the *B. bovis* RAP-1. Sequence alignment of both ORFs is shown in Fig. 1A. The regions of sequence identity amongst these 2 ORFs are restricted to the first 300 amino acids of the proteins and they include the strict conservation of 4 cysteine residues (Fig. 1A). Additionally, there is partial conservation of other RAP-1 signature motifs, such as the 14-mer sequence (Suarez *et al*. 1991*a,b*) (Fig. 1A), while RRA lacks the series of repeats present in the C-terminal end of the *B. bovis* RAP-1 (Suarez *et al*. 1991*a*). Both *rap-1* genes and the *rra* gene are located on chromosome 4. However, the *rra* gene is located approximately 90 kbp downstream from the *rap-1* locus, thus not closely linked in the *B. bovis* genome (Brayton *et al*. 2007). The *rra* gene is not flanked by other *rap-1* related or any predicted surface protein sequences. Phylogenetic analysis of all known RAP-1 sequences from *Babesia,* using MSA-1 as an outlier (Fig. 1B) shows 2 major clusters of RAP-1 sequences, with one of the branches containing RAP-1b of *B. bigemina* and RRA, suggesting that RRA is more closely related to the RAP-1b sequence from *B. bigemina* than to *B. bovis* RAP-1. Blast searching of the T2Bo genome using the RRA sequence indicates that this strain contains a single gene copy of the *rra* gene. To determine whether the RRA sequence is conserved among geographically distinct *B. bovis* strains, we compared the sequences of the *rra* gene in the T2Bo (Texas) and the Mo7 strains with *rra*-PCR amplicons obtained from the R1A and S2P strains from Argentina. The predicted amino acid sequence of RRA is strictly conserved (100% identity) among all strains analysed (data not shown), thus similar to RAP-1, RRA appears to be highly conserved among distinct geographical isolates of *B. bovis*.

Expression in Babesia bovis *merozoites and immunogenicity of RRA*

We initially analysed the presence of *rra* transcripts in cultured merozoites by Northern blot analysis on total RNA from *B. bovis* Mo7 merozoites, using *rap-1* and *rra* specific probes. Intense hybridization bands were evident in the Northern blot using the *rap-1* specific probe whereas a weaker signal was detected using the *rra* probe (red box, Fig. 2A). In control dot blots, the 2 dig-labelled probes generated comparable signals (data not shown).

To confirm transcription of the *rra* gene in *B. bovis* merozoites and to estimate relative amounts of *rap-1* and *rra* transcripts, RT-PCR was performed using either 1 μg or 0·02 μg of total *B. bovis* merozoite RNA using *rap-1* and *rra* specific primers (Fig. 2B). A stronger ~1·7 kbp band was obtained when using control *rap-1* specific primers in RT-PCR amplification experiments on 1 μg of total RNA compared to the ~1 kbp *rra* amplicon (Fig. 2B). However, when using 50-fold diluted total RNA (0·02 μg), no amplification products were obtained for the *rra* transcript, whereas the ~1·7 kbp *rap-1* transcript was still amplified. The ~1·0 kbp RT-PCR product obtained for *rra* was identical in size to the product of PCR amplification of genomic DNA using identical sets of primers (Fig. 2B). No amplification product was obtained when the RT was omitted in the RNA PCR amplifications, or when the reaction was performed in the absence of genomic DNA (Fig. 2B). Sequencing of the *rra*-RT-PCR amplification product showed 100% identity with the *rra* gene. Thus, taken together, these results confirmed that cultured *B. bovis* merozoites contain *rra* transcripts, but the data suggest that *rra* transcripts are relatively less abundant than *rap-1* transcripts.

To confirm expression of the RRA protein in *B. bovis* merozoites, we performed immunoprecipitation of ^{35}S metabolically labelled parasite proteins using rabbit antibodies prepared against sRRA-1, a synthetic peptide representing putative antigenic regions of RRA. The rabbit anti- sRRA-1 peptide antibodies precipitated a metabolically labelled *B. bovis* antigen of ~43 kDa (Fig. 2C, lane 4), a size that is consistent with the predicted molecular weight of the RRA ORF. Immunoprecipitation of RAP-1 with anti-RAP-1 monoclonal antibody BABB75A4 is shown as a control on lane 2. No labelled products were precipitated by monoclonal control antibody or by rabbit pre-immune sera (lanes 1 and 3 respectively).

Fixed IFA using the rabbit antibodies directed against the synthetic sRRA-1 peptide and with the monoclonal antibody BABB75A4 reactive with RAP-1 are shown in Fig. 3. IFA revealed reactivity of the anti-sRRA-1 antibodies with *B. bovis*-infected erythrocytes only when incubated at a low antiserum dilution (1:10). Higher dilutions of the antiserum (1:100) failed to show significant fluorescence (not shown). Taken together, the transcription analysis, immunoblot and IFA data show that the RRA is expressed in *B. bovis* merozoites. Additionally, the results suggest that RRA is present at lower levels when compared to RAP-1 in cultured merozoites.

We further tested the ability of sera from a *B. bovis* hyperimmune cow to recognize recombinant RRA in Western blots, which showed a faint band at the correct molecular weight (Fig. 4A, lane 4). This result also confirms RRA expression by *B. bovis*
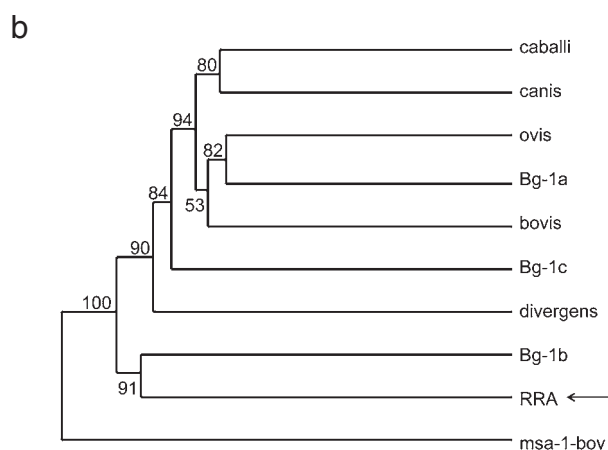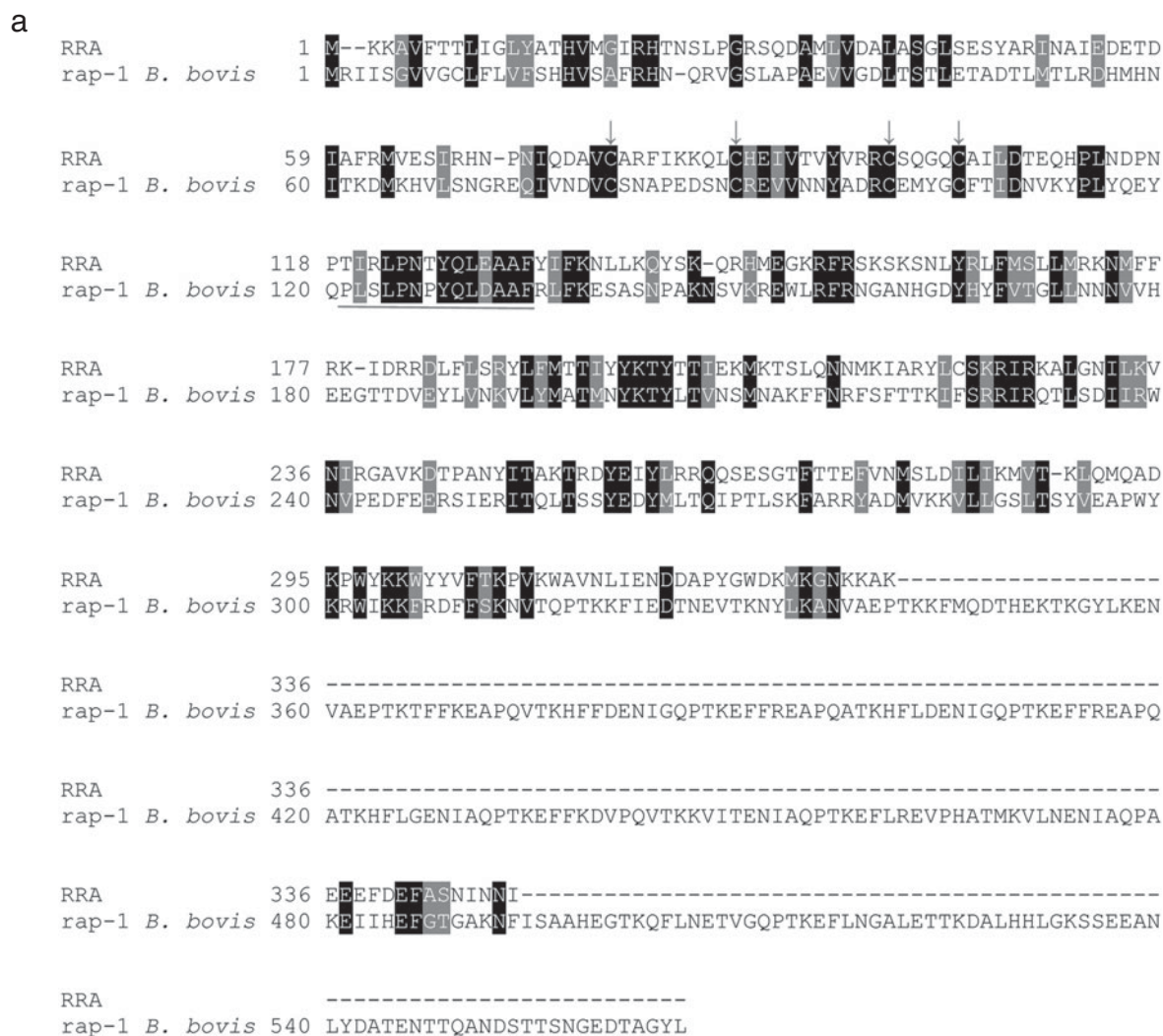
Fig. 1. (A) Sequence comparison between RAP-1 and RRA from *Babesia bovis*. Conserved cysteine residues are indicated with red arrows. The 14 amino acid RAP-1 conserved region among RAP-1 and RRA (14mer) is underlined. (B) Phylogram of the amino acid sequences from all known ORFs derived from *rap-1* gene family of *B. bovis*, *B. divergens, B. canis, B. ovis* and *B. bigemina*, produced using the program Phylip with bootstrap values indicated at each branch point. The *B. bovis* MSA-1 amino acid sequence was used as an outlier. The most parsimonious tree is shown. GenBank Accession numbers are: *B. bovis rra*: XP_001610950.1; *B. bovis rap-1* (bovis): AAB84267; *B. caballi rap-1* (caballi): BAA83725; *B. divergens rap-1* (divergens): Z49818; *B. canis rap-1* (canis): CAA01285; *B. ovis rap-1* (ovis): AAA27805; *B. bigemina rap-1a*-p58 (Bg-1a): 1906304A; *B. bigemina rap-1b* (Bg-1b): AAB72094; *B. bigemina rap-1c* (Bg-1c): AAN84523; and *B. bovis msa-1* (msa-1-bov): AAK07773.
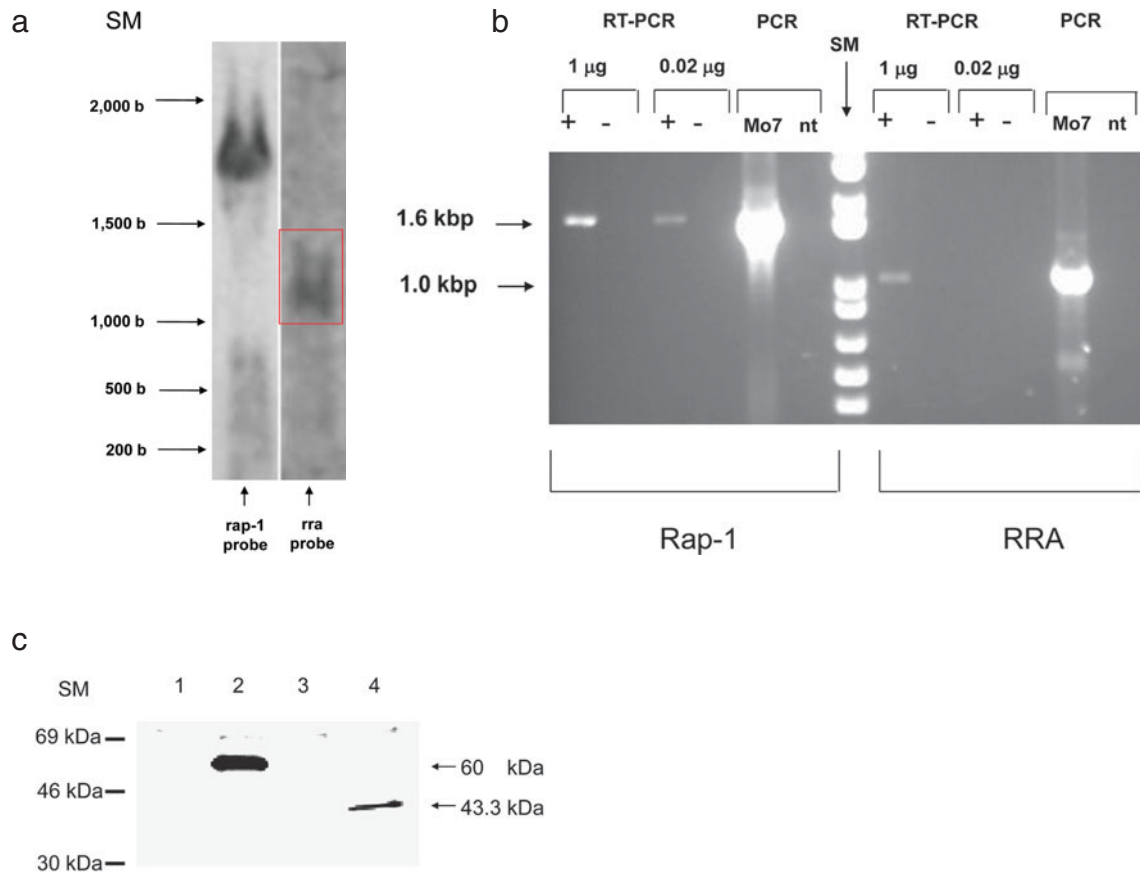
Fig. 2. Expression of RRA. (A). Northern blot analysis. Total RNA extracted from *Babesia bovis* Mo7 strain merozoites was separated in a 2% denaturing agarose gel, transferred to a Nylon membrane and hybridized with dig-labelled *rap-1* (left panel) and *rra* (right panel) probes. Size markers in base numbers are marked on the left. (B) RT-PCR amplification of the *rap-1* and *rra* ORFs from total RNA extracted from *B. bovis* Mo7 strain merozoites and control PCR amplifications of genomic DNA from *B. bovis* Mo7 strain merozoites, and no template (nt). +: reverse transcriptase reactions;-: control reactions without reverse transcriptase. The set of primers used for each PCR reaction is indicated at the bottom. Size markers (SM) in base pairs are indicated on the left. (C) Immunoprecipitation of [^{35}S]-methionine metabolically labelled *B. bovis* proteins with negative control mouse monoclonal antibody Tryp (lane 1), monoclonal antibody BABB75 against RAP-1 (lane 2), control pre-immune rabbit serum (lane 3), and serum from a rabbit immunized with sRRA-1, a synthetic peptide representing putative B-cell epitopes of RRA (lane 4). Position of size markers (expressed in kDa) are indicated on the left. The size of the immunoprecipitated proteins is shown on the right.

parasite stages that occur in infected cattle. However, additional immunoblots performed with 3 sera from naturally infected cattle that recognized RAP-1, failed to recognize recombinant RRA (data not shown).

To further analyse CD4 T-cell responses to the RRA, we tested the ability of the recombinant RRA to stimulate *B. bovis*-immune T-cells *ex vivo*. As shown in Fig. 4B, no significant stimulation was obtained using recombinant RRA as antigen whereas both *B. bovis* RAP-1 and crude parasite membrane antigens strongly stimulated T-cell proliferation in the same assay. Collectively, these data suggest that in contrast to RAP-1, RRA acts as a subdominant antigen during *in vivo* infection of cattle. However, T-cells from only 1 *B. bovis* immune animal were available for testing in this study, and further analysis of a larger population of *B. bovis* immune cattle is required.

The RRA contains neutralization-sensitive epitopes

To determine the possible functional relevance of RRA, we tested whether rabbit antibodies against sRRA-1 containing predicted B-cell epitopes that were shown to immunoprecipitate a native ~ 43 kDa antigen, could inhibit *B. bovis* infection of erythrocytes using *in vitro* neutralization assays. Incubation of free merozoites with rabbit antiserum specific for sRRA-1 resulted in a statistically significant decrease of PPE of *in vitro* cultures when compared with pre-immune rabbit serum, with P values of 0·00236 and 0·0113 at 3 and 4 days of culture, respectively (Fig. 5). When comparing medium with antiserum specific for sRRP-1, there was a significant difference at day 4, with a P value of 0·00056, but not at day 3 ($P = 0·03$). These results suggest that the sequence in the synthetic peptide contains B-cell epitopes that are neutralization-sensitive and support that, similar to
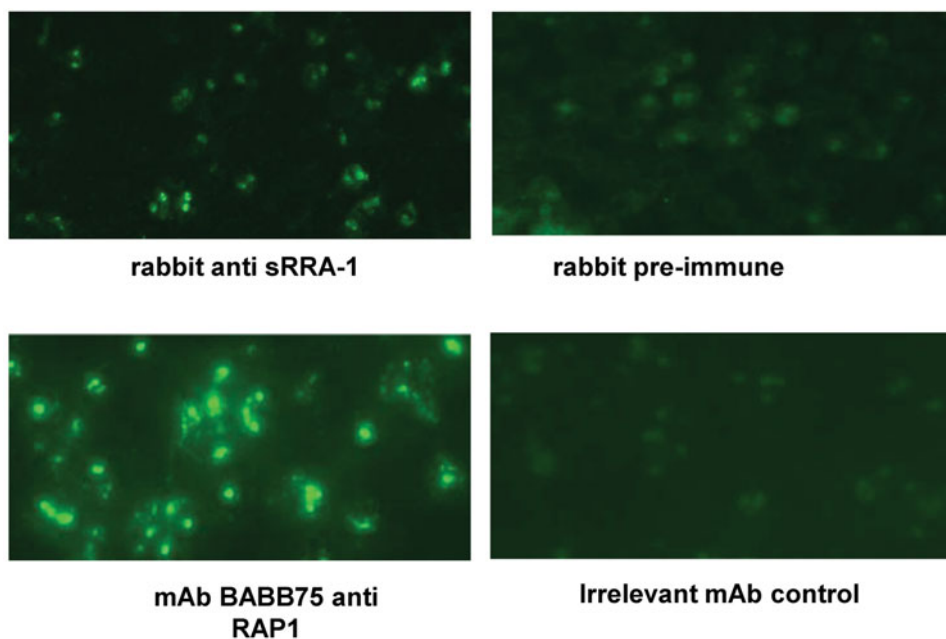
Fig. 3. Immunofluorescence analysis of *Babesia bovis* Mo7 merozoites in bovine erythrocyte culture using anti sRRA-1 rabbit antibodies (top left panel), or control pre-immune rabbit serum (top right panel) diluted 1:10, and anti-RAP-1 monoclonal antibody BABB75A4 (bottom left panel) or control monoclonal antibody Tryp (bottom right panel) used at 20 µg/ml.

RAP-1, RRA might play an important role in the process of invasion of red blood cells.

DISCUSSION

A gene that codes for a RAP-1-like protein was identified in the *B. bovis* genome and termed RAP-1 related antigen (RRA). Interestingly there are parallels between the RRA of *B. bovis* and its putative orthologue in *B. bigemina*, RAP-1b (Suarez *et al*. 2003). However, in contrast to what was previously found in *B. bigemina* where multiple copies of tandemly arranged *rap-1b* genes are present (Suarez *et al*. 2003), the *rra* gene of *B. bovis* is present as a single copy and it is not closely linked to the *rap-1* locus.

Similar to *rra*, *B. bigemina rap-1b* transcripts are relatively less abundant than *rap-1a*, and *rap-1b* transcripts are either not translated or RAP-1b protein is present in undetectable amounts in *B. bigemina* merozoites. In addition, both recombinant versions of *B. bovis* RRA and *B. bigemina* RAP-1b failed to stimulate proliferation of T-cells either from *B. bovis* or *B. bigemina* immune cattle (data presented in this study and W. Brown, personal communication, and Brown and Palmer, 1999). It is possible that the inability of RRA to stimulate T-cells in our proliferation experiment is as a result of the low level of expression of RRA in merozoites during bovine infection, which may be insufficient to induce a detectable level of T-cell response. Alternatively, the amount of RRA in the merozoite membrane extract used for establishing the T-cell line might be too low to stimulate the growth of RRA-specific T cells in

culture. Additionally, sequence comparison demonstrates that all T-cell epitopes mapped in *B. bovis* RAP-1 using CD4+ T cells from animal C97, which was also used in the current study, are poorly conserved or absent in RRA (Norimine *et al*. 2002). Lack of conservation of these epitopes could also explain the lack of response of animal C97 T cells to RRA.

Interestingly, previous experiments demonstrated that (1) immunization with RAP-1 or an N-terminal truncated version of RAP-1 (RAP-1-NT) conserved region is not sufficient to elicit protective immune responses despite strong immunological stimulation (Norimine *et al*. 2003); (2) RAP-1 is an immunodominant, monomorphic antigen, and cattle remain persistently infected despite high antibody titres against RAP-1 (Brown *et al*. 1996; Suarez *et al*. 1998; Boonchit *et al*. 2002), and (3) anti-RAP-1 antibodies can only partially neutralize erythrocyte invasion by both *B. bovis* merozoites and sporozoites (Yokoyama *et al*. 2002; Mosqueda *et al*. 2002). Consistently, in this study we also observed strong but incomplete neutralization effects of *B. bovis* merozoites using anti-sRRA-1 antibody in *in vitro* neutralization assays. Although the exact functional roles of RAP-1 and RRA remain unknown, the partial antibody-mediated neutralization effects of anti-RAP-1 antibodies supports that an additional RAP-1-like molecule may be used for *B. bovis* survival in the phase of strong anti-RAP-1 immune response in infected cattle. Conservation of the RAP-1 signature motifs in RRA supports functional roles common between these two molecules. However, the T-cell proliferation assay and the weak antibody
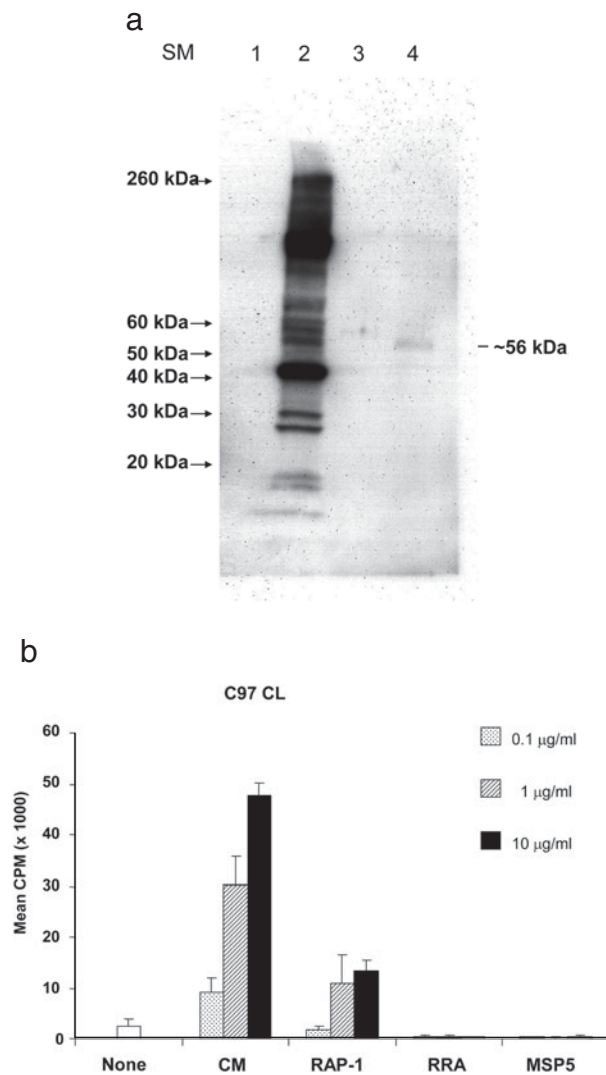
a

b

Fig. 4. Immunogenicity of RRA. (A) Antibodies from hyperimmune *Babesia bovis* specific serum weakly recognize recombinant RRA in a Western blot. Lane 1, uninfected erythrocytes. Lane 2, *B. bovis*-infected erythrocytes. Lane 3, irrelevant recombinant control protein rAm780. Lane 4, recombinant RRA. The locations of the size markers (SM) are indicated on the left, and the calculated size of the product recognized by antibodies is indicated on the right with an arrow. (B). T-cell stimulation. A short-term T-cell line (CL) was generated by stimulating peripheral blood mononuclear cells derived from a *B. bovis*-immune animal C97 with *B. bovis* membrane- and organelle-enriched pellet (CM) for 1 week and rested for 1 week. The proliferation assay was carried out using 3 concentrations of CM, recombinant RAP-1, recombinant RRA, and negative control recombinant MSP-5 from *A. marginale*. Results are expressed as mean cpm (counts per minute) incorporated into 3 parallel cultures +/− S.D.

response suggest that, in contrast to RAP-1, RRA is poorly antigenic during infection. Thus collectively, the data suggest that RRA is expressed in relatively small amounts and is therefore unable to elicit significant immunity in infected cattle, but yet it may play a role in parasite invasion and survival.
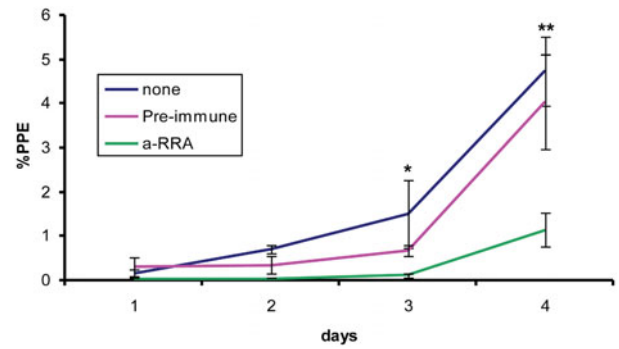

Fig. 5. *In vitro* growth curves. *Babesia bovis* strain Mo7 merozoites were exposed either to pre-immune rabbit serum, anti-sRRA-1 peptide rabbit serum, or no sera, and incubated with bovine erythrocytes and cultured for 4 days, as described in the Materials and Methods section. PPE in each culture well were calculated every 24 h. The data show the mean ± 1S.D. of the percentage of infected erythrocytes in 3 independent assays. $*P < 0.025$ for control *vs* anti-rRRP-1 sera; $**P < 0.025$ for all comparisons.

Therefore, because it is a weak immunogen during infection yet potentially important for erythrocyte invasion (as demonstrated in *in vitro* neutralization assays), we hypothesize that RRA may provide alternative means for the parasite to escape from anti-RAP-1 antibody-mediated neutralization. This could explain the lack of total inhibition of invasion observed in the *in vitro* neutralization studies using either anti RAP-1 or RRA antibodies. Furthermore, as immunodominant antigens such as RAP-1 (Norimine *et al.* 2003) and MSA-1 (Hines *et al.* 1995) fail to induce protective immunity, subdominant antigens like RRA may be better candidates for developing vaccines which could potentially elicit a neutralizing antibody response *in vivo* (Brown *et al.* 2006). In this scenario, it is also possible to speculate that the immunodominant RAP-1 molecule acts as a decoy, diverting potential immune responses against other functionally relevant molecules required for invasion by the parasite such as RRA, thus assuring persistent infection of cattle by *B. bovis*.

In summary, a previously unreported and highly conserved RAP-1-related protein encoded by *B. bovis* is expressed during infection of the bovine host. Based on the conservation of the RAP-1 signature motifs in RRA, we hypothesize that RRA is a subdominant functional equivalent of RAP-1 that is expressed at a low level in *B. bovis* merozoites. Thus it is possible that expression of RRA is strongly regulated in order to minimize host immunity during infection and contribute to persistent infection. This feature could provide a useful mechanism for the parasite to escape the immune response directed against highly immunogenic antigens such as RAP-1 as well as be a new target for immunization strategies. The apparent low level of expression and the subdominant characteristics of the *B. bovis* RRA

raise interesting questions. The possible functional relationships of RRA and RAP-1, whether the expression of RRA is differentially regulated during the *B. bovis* life cycle, and its possible role in erythrocyte invasion and in the induction of protective immune responses need to be addressed in future studies.

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