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Proline racemases are conserved mitogens: Characterization of a *Trypanosoma vivax* proline racemase[☆]

Nathalie Chamond, Alain Cosson, Nicolas Coatnoan, Paola Minoprio*

Institut Pasteur, Laboratoire d'Immunobiologie des Infections à *Trypanosoma*, Département d'Immunologie, 25 rue de Dr Roux, Paris 75724, France

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ABSTRACT

Trypanosoma cruzi proline racemases (TcPRAC) are the only eukaryotic proline racemases described so far. Except their role in the interconversion of free L- and D-proline enantiomers, parasite TcPRACs are involved in major *T. cruzi* biological pathways. These essential enzymes are implicated in the process of parasite differentiation and the acquisition of virulence during metacyclogenesis and are currently considered as key targets for drug development against Chagas' disease. In this study, we searched for the presence of TcPRAC gene homologues among other trypanosomatid genomes. Despite the high degree of gene synteny observed in Kinetoplastidae genomes, PRAC genes are missing in *Trypanosoma brucei*, *Trypanosoma congolense* and *Leishmania* spp. genomes. Interestingly, we identified a hypothetical PRAC gene in *Trypanosoma vivax* that is the major hemoparasite responsible for livestock trypanosomiasis, a serious economical impact for most of African and South American countries. We report here that the product of this *T. vivax* gene is *bona fide* a proline racemase with an activity comparable to the one we described previously for TcPRAC. Inhibition studies using the pyrrole-2-carboxylic acid confirmed that this compound is a competitive inhibitor for both TcPRAC and TvPRAC enzymes. Similarly to TcPRAC and all members of the racemase family studied so far in other pathogenic and nosocomial bacteria, our results show that TvPRAC is a T-cell-independent B-cell mitogen. Therefore the product of the novel TvPRAC gene identified in *T. vivax* and reported herein has the potential to be used as a drug target for this parasite-based trypanosomiasis.

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1. Introduction

Human sleeping sickness, leishmaniasis and Chagas' disease are vector-borne diseases that threaten the lives of millions of individuals in African and South American countries. Today's challenges are to strengthen surveillance in endemic areas and sustained efforts are undertaken to eliminate these most neglected diseases, which are all caused by distinct parasites of the *Trypanosomatidae* family.

Basic research on the intricacies of trypanosomatids biology during development in their different hosts are currently aiming to reveal some common and essential biochemical pathways and consequently to define new promising targets for the design of new therapies. However, trypanosomatids have evolved complex life cycles involving very diverse blood-sucking insect vectors and vertebrate hosts. These parasites employ diverse immune evasion strategies and interact with a number of target tissues of their hosts,

causing very different pathologies. Interestingly, despite species diversion dated millions of years ago, genomes of trypanosomatids are highly syntenic, featuring about six thousand genes in common [1]. Furthermore, these protozoa share many general characteristics such as polycistronic transcription [2] and conservation of their intracellular sub-cellular structures, i.e. the kinetoplast [3], the acidocalcisome [4] and the glycosome [5]. Along with conserved core parasite processes, all trypanosomatids are able to adapt to the available carbon sources present in their hosts for their energy metabolism. While within their vertebrate hosts, *Trypanosoma brucei* and *Trypanosoma cruzi* exhibit a marked preference for glucose [6,7], *Leishmania* spp. use mainly oxidized sugars and fatty acids as carbon sources [8]. At the insect stages, they make use of amino acid catabolism for energy production, mostly of L-proline, L-threonine and L-alanine [9–12]. L-Proline is actually of the utmost relevance for trypanosomes as a source of energy [7,13], but may also operate as an osmoregulator [14,15] and as a significant signal for differentiation from a non-infective to an infective parasite form [16,17]. The significance of proline in trypanosomatid metabolic reactions was strengthened by the identification of proline transporters in *T. brucei* [18], *T. cruzi* [19] and *Leishmania donovani* [20–22] and by the demonstration of a functional proline racemase in *T. cruzi* (TcPRAC) that is differentially expressed in all stages of *T. cruzi*

[☆] Note: Nucleotide sequence data reported in this paper for *Trypanosoma vivax* proline racemase (TvPRAC) are available in the GenBank™, under the accession number EF175213.

* Corresponding author. Tel.: +33 1 45688615; fax: +33 1 40613185.
E-mail address: pmm@pasteur.fr (P. Minoprio).

development. One enzyme isoform, released by the infective forms of the parasite, triggers non-specific polyclonal B-cell responses in the host, contributing to mechanisms of parasite escape from the host immune system [23]. Additional studies revealed that *TcPRAC* genes are essential and participate in key metabolic pathways during metacyclogenesis. Indeed the viability of mutant parasites is severely compromised by gene knock down, while parasites over expressing *TcPRAC* gene exhibit increased virulence toward host cells [24].

Because proline racemases (PRAC) catalyze the interconversion of L- and D-proline enantiomers, it was previously postulated that these enzymes could be involved in environmental sensing by controlling the availability of free L-/D-proline. This in turn would regulate important metabolic pathways in intracellular and/or extracellular parasite compartments [25]. We used a previously defined signature for proline racemases and available genomic databases to explore for homologues of *TcPRAC* genes. Whereas several bacterial species do possess functional *PRAC* or resembling hydroxyproline epimerase genes [26], only one *PRAC* homologue was identified among trypanosomatids, notably *Trypanosoma vivax*. This major livestock trypanosome is cyclically transmitted between domestic and wild ruminants by tsetse flies, tabanids and other varieties of biting flies.

Despite the prevalence and the incidence of *T. vivax* trypanosomiasis in the new world and its economical impact [27–29], there have been very few investigations on the immunobiology and the pathogenesis of the infectious process induced by this parasite in mammalian hosts. In the present study, we report that *T. vivax* possesses a proline racemase gene (*TvPRAC*) that encodes a functional proline racemase. The biochemical characterization of the corresponding protein revealed that *TvPRAC* exhibits characteristics and kinetic parameters comparable to *T. cruzi* *PRAC*. In addition, the dissociation constant (K_i) of the enzyme-inhibitor complex obtained with the pyrrole-2-carboxylic acid (PYC), a specific inhibitor of proline racemases, is equivalent to that found for *TcPRAC* enzymes. We have also demonstrated both *in vitro* and *in vivo* that *TvPRAC* displays mitogenic properties to host B-cells. Although African and American trypanosomes seem to interact fairly differently with the immune system of their hosts and therefore to engage an array of evasion strategies, the presence of intracellular and/or the release of parasite proline racemases might *per se* introduce novel alternatives to a better understanding of the mechanisms involved in trypanosomiasis immunopathogenesis.

2. Materials and methods

2.1. Parasites and soluble extracts

Epimastigote forms of *T. cruzi* (clone CL brener F11F5) are maintained by weekly passage in LIT medium (www.pasteur.fr/recherche/unites/tcruzi/minoprio/TcruziDB/clbrener.html). *T. vivax* (stock ILRAD 1392), initially isolated from a cow in Nigeria [30], was kindly provided by R. Brun (Swiss Tropical Institute, Basel, Switzerland). Bloodstream forms of *T. vivax* are maintained by passage every 7 days in Swiss mice. Bloodstream (strain Antat1) and procyclic forms of *Trypanosoma brucei* (strain 427) were a kind gift of P. Bastin and I. Subota (Institut Pasteur, UP *Trypanosoma* Cell Biology, Paris, France). Procyclic forms of *Trypanosoma congolense*, strain TREU183: 29-13, were obtained from F. Bringaud (UMR 5536 CNRS, Université Victor Segalen Bordeaux 2, France) and kept in culture in MEM supplemented medium, as described [31]. Bloodstream forms of *T. congolense*, clone IL-3000, that induces an acute infection in BALB/c mice and severe infections in bovine, were obtained from Baltz (UMR 5534 CNRS, Université Victor Segalen Bordeaux 2, France), as described [32]. Parasite soluble extracts were prepared by three repeated cycles of freeze/thaw, followed by 30 min of son-

ication at 4 °C. Extracts were cleared by centrifugation (4 min at 14,000 rpm) and the amount of soluble proteins quantified.

2.2. RT-PCR, Southern and Northern blots

TvHYP1 probe was obtained by PCR amplification of *T. vivax* DNA with *TvNcoF* forward (5'-GGCGCCATGGAGTTCACCGGAACAATG-3') and *TvBamR* reverse (5'-CCGGGATCCACGCTCAGCGTAAAGCC-3') primers and then labelled with [α -³²P]-dCTP using megaprime DNA labeling system (Amersham Life Science). Blots were hybridized overnight in ULTRAhyb™ solution (Ambion) at 42 °C and washed in 2× SSC/0.1% SDS at 42 °C, 2× SSC/0.1% SDS at 55 °C and 2× SSC/0.1% SDS at 60 °C. Autoradiography was performed by overnight exposure of blots on BioMax MS-1 films (Eastman Kodak). RNA from *T. cruzi* metacyclic forms or *T. vivax* bloodstream parasites was extracted using the RNeasy kit (Qiagen) following the manufacturer's instructions. 1.2% agarose-formaldehyde gels were loaded with 5 µg of total RNA and run overnight in 1× MOPS buffer, pH 7.0. Gels were stained with ethidium bromide and blotted onto a Hybond uncharged membrane in 10× SSC. After transfer, the membrane was rinsed in 10× SSC, air-dried and exposed to UV light for 8 min. Hybridization was performed using a *TvHYP1* probe prepared as described above, in 2× Denhardt's/6× SSC/0.1% SDS overnight at 68 °C. The membrane was then washed three times at 68 °C in 0.2× SSC/0.1% SDS, and autoradiography was performed overnight, followed by a 4-day long exposure of blots on BioMax MS-1 films (Eastman Kodak). Reverse transcription was performed with Superscript II (Invitrogen) using the antisense Oligo d(T)_{12–18} Primer (Invitrogen) and corresponding cDNAs amplified by PCR using Phusion Polymerase and the following primers: *TvSL*: 5'-AGAACAGTTTCTGTACTATATTG-3', *SL-all*: 5'-TRRWACAGTTTCTGTACTATATTG-3', *TvBamR* or *JOJOR* 5'-AARAAAYTRCCWCCRAAKG-3'. The obtained fragments were used for cloning into pCR-Blunt II-TOPO vector using the Zero blunt TOPO PCR cloning kit (Invitrogen) following the manufacturer's instructions. Recombinant clones were screened and sequenced.

2.3. Western blots

Recombinant *TcPRAC* and *rTvPRAC* were separated by gradient SDS-PAGE (4–12%) and proteins were electrophoretically transferred onto nitrocellulose membranes. Membranes were saturated in Tris-buffered saline and low-fat milk, washed, incubated with anti-HisTag monoclonal antibody (Novagen), washed and developed with peroxidase-labelled secondary antibody using chemiluminescence (ECL Kit, Amersham).

2.4. Plasmid construction and protein purification

TvPRAC gene was obtained by PCR using *TvNcoF* and *TvBamR* primers, and cloned into *NcoI*/*BamHI* sites of pET28b(+) expression vector (Novagen/Merck) using the Rapid Ligation Kit (Roche). *Escherichia coli* DH5 α cells were transformed with empty or ligated plasmids. Plasmids were extracted with the Qiaprep Spin Miniprep Kit (Qiagen) from bacterial pellets from individual colony cultures and sequenced (Genome express, Meylan/France). Sequence, ORF and the presence of C-terminal 6×-His Tag were verified. *E. coli* BL21 (DE3) cells were transformed and recombinant proteins were purified as described [25].

2.5. Racemization assays

The percentage of racemization with different concentrations of L-proline, D-proline, L-alanine, L-valine and L-hydroxy (OH)-proline were calculated as described in Ref. [23] by incubating a 500 µl mixture of 10 µg of recombinant protein or soluble parasites extracts

and 40 mM substrate in 0.2 M sodium acetate, pH 6.0, for 30 min at 37 °C. The reaction was stopped by freezing. Water (1 ml) was then added and the optical rotation was measured in a polarimeter 241 MC (PerkinElmer Life Sciences) at a wavelength of 365 nm, in a cell with a path length of 10 cm, at a precision of 0.001°. The percent of racemization of 40 mM L-proline as a function of pH was determined using 0.2 M sodium acetate, potassium phosphate, and Tris–HCl buffers; reactions were incubated 30 min at 37 °C as described above. All reagents were purchased from Sigma.

2.6. Kinetic assays

Concentrations of L- and D-proline were determined polarimetrically from the optical rotation of the solution at 365 nm in a cell of 10-cm path length, thermostated at 37 °C. Preliminary assays were done with 20 µg/ml of recombinant protein, 40 mM L-proline in 0.2 M sodium acetate, pH 6.0, in a final volume of 1.5 ml. Optical rotation was measured every 5 s during 10 min. After the determination of the linear part of the curve, velocity with 20–160 mM of substrate was measured every 30 s during 10 min to determine K_m and V_{max} . Calculations were done using Kaleidagraph® program and Michaelis–Menten equation. Inhibition assays were performed by incubating 20 µg/ml of recombinant protein, 1–100 µM PYC, 40 mM L-proline as described above. Graphic representation and Lineweaver–Burk linear curve regression allowed the determination of K_i as $[1]/[(\text{apparent } K_m/K_m) - 1]$. All reagents were purchased from Sigma.

2.7. Assays of proliferative activity

In vitro proliferation was accomplished using freshly recovered splenocytes from euthymic or athymic Swiss mice seeded at a density of 2.5×10^5 cells/well and incubated for 24, 48, 72 and 96 h with increasing concentrations of recombinant protein (0.8–100 µg/ml) or with concanavalin A (2.5 µg/ml) and liposaccharide (5 µg/ml) mitogens in 5% FCS in RPMI-1640 complete medium. Polymyxine B was used at a final concentration of 2 µg/ml. PYC inhibition was performed by pre-incubating a solution of 1 mg/ml of TvPRAC with 10 mM PYC for 30 min at 37 °C. Cultures were kept at 37 °C in 5% CO₂ and harvested after a 16-h pulse of 1 µCi/well ³H-thymidine. ³H-thymidine uptake was determined using a beta-counter 1450 Microbeta Trilux (PerkinElmer). All data were obtained in triplicate and the corresponding standard deviation was calculated.

2.8. In vivo B-cell assays

Naïve Swiss mice were injected with 50 µg of TvPRAC. Splenocytes were recovered at day 0, 2 or 5 post-injection, the number of cells was recorded individually, the levels of seric IgG and IgM antibodies determined by ELISA and the number of Ig-producing B-cells was determined by ELISPOT, as described before [33].

3. Results

3.1. Locus analysis of the PRAC environment

Previous studies of *T. brucei*, *Leishmania major* and *T. cruzi* genomes revealed significant conservation of gene orders and consequently a high degree of gene synteny [1]. Therefore, several megabase pairs of syntenic blocks were identified between these three kinetoplastids. By comparing numbers of non-synonymous mutations with non-synonymous sites (d_N) of these genomes, followed by categorization of the genes by gene ontology, low d_N values have been found for genes coding for metabolism, cell growth and cell preservation. This would reflect the existence of core processes common to those three parasites [1]. Given that

TcPRAC seems to be involved in key developmental processes of *T. cruzi*, we hypothesized that proline racemases could as well be present – and share similar biological functions – in other trypanosomatids. To verify this hypothesis we first performed BLAST searches on the available *Kinetoplastidae* genome databases with full-length TcPRAC sequences. This provided several hits in *T. brucei*, *T. vivax*, *T. congolense*, as well as *Leishmania* spp. genomes (see Table 1). Although most of the retrieved sequences presented the previously described MCGH and/or MIII* motifs [25], they have a greater identity with bacterial proteins than with *T. cruzi* PRAC. These hits most likely are bacterial genome DNA contaminants and were discarded. However, one *T. vivax* sequence displayed a 59% identity as well as a compatible score with TcPRAC, suggesting the presence of a hypothetical PRAC gene in this parasite.

In order to confirm the lack of PRAC homologues in the other members of the family, we explored the PRAC locus genetic environment in *T. cruzi*. We verified whether its flanking regions were conserved in other African trypanosomes and *Leishmania* spp. regardless of the presence or absence of PRAC gene. As shown in Fig. 1 A the PRAC locus is well conserved in both *T. cruzi* and *T. vivax* genomes corroborating the high degree of synteny for this region between these two species. Interestingly, the analysis of *T. congolense* genome revealed that comparably to *T. cruzi* and *T. vivax* the PRAC locus is flanked by genes encoding “cold shock” (CS) and “hypothetical” (H) proteins. Nevertheless the region enfolds only remnants of a PRAC sequence which are not compatible with the expression of a functional gene product and could thus be considered as a pseudogene. While the genome of two sub-species of *T. brucei* exhibit the same PRAC genomic context and conserved gene order as *T. cruzi*, *T. vivax* and *T. congolense*, specific deletion of the entire PRAC gene seems to have occurred within this particular syntenic block. Further PCR analysis of the region using *T. brucei* genomic DNA and specific CS and H primer sequences confirmed the presence of a shorter sequence incompatible with the size of the PRAC gene (not shown). An interesting example of localized chromosomal rearrangement can be seen in three species of *Leishmania*, where a Tubby protein-like (*Tb*) gene sequence is observed in place of a PRAC gene, similarly flanked by CS and conserved H genes. However the contiguous positioning to enolase (*Enol*) sequences of this *Leishmania* locus is different than trypanosome genomes. In addition, a syntenic breakpoint is observed in *Leishmania braziliensis*, given that a long segment is inserted across H and *Enol*, which is homologous to a *L. major* block located on another chromosome. The remaining neighbouring sequences of this locus environment are preserved in all species analyzed. The phylogenetic analysis presented in Fig. 1 B suggests that PRAC gene could have been inactivated prior the separation of *T. brucei* and *T. congolense* clades and underwent further deletion in the *T. brucei* branch. To confirm these observations, we produced total RNA from different forms of *T. brucei*, *T. congolense*, *T. vivax* and *T. cruzi* for RT-PCR analysis. Oligo dT-first strand cDNA was obtained and PCR reactions performed using a spliced leader (SL) oligonucleotide sequence common to all species (SL-all) and a reverse primer (JOJOR) common to *T. cruzi*, *T. vivax* and *T. congolense* corresponding sequences. Resulting fragments were hybridized with a MCGH-specific probe, embedding one of the PRAC catalytic residues. As anticipated, while fragments of approximately 600 bp were obtained for *T. cruzi* and *T. vivax*, no hybridization was observed for *T. brucei* or *T. congolense* amplification products (data not shown).

3.2. Molecular characterization and in vivo expression of the putative TvPRAC gene

To investigate the genomic organization and transcription of the putative PRAC gene in *T. vivax*, we obtained the full-length of the identified TvHYPI (tviv1929b09.p1k.8) gene sequence by PCR-

Table 1
Database collection from *in silico* searches and complementary information on sequences.

Organism	SeqID ^a	Positives ^b (%)	Identities ^c (%)	Closest spp. ^d	Score ^e
<i>T. brucei</i> 927	telo24Ac10.p1ka	58	41	<i>P. fluorescens</i>	0.0
<i>T. brucei</i>	tryp IXb-28b06.q1c	59	42	<i>B. cereus</i>	3e–173
	tryp IXb-28b06.p1c	58	42	<i>B. cereus</i>	8e–174
<i>T. congolense</i>	congo208e06.p1kw	30	50	<i>P. putida</i>	1e–128
<i>T. vivax</i>	tviv1929b09.p1k 8	75	59	<i>T. cruzi</i>	1e–37
<i>L. major</i>	LM16 BIN contig2054	51	32	<i>P. fluorescens</i>	0.0

^a Sequences were obtained by blasting *TcPRAC* against gene DB and the encoded proteins were aligned using the BLAST 2 protein tool.
^b Percent of similar residues.
^c Percent of identical residues.
^d Best hit of the same sequences when blasted against the NCBI database.
^e The corresponding score.

assisted amplification of *T. vivax* genomic DNA using *TvNcoF* and *TvBamR* primers (see Fig. 2). Total RNA from *T. vivax* bloodstream trypomastigotes was further analyzed by Northern blot hybridized with *TvHYP1* probe. As expected a unique *T. vivax* transcript of around 1.5 kb has been amplified from the genomic sequence (Fig. 2A). In contrast specific *TcPRAC* probe hybridizes with two *T. cruzi* RNA transcripts corresponding to *TcPRACA* and *TcPRACB*

homologous gene copies, known to migrate near by the smallest 1.4 kb ribosomal RNA [25] (Fig. 2A). Reverse transcription PCR, using primers specific for the SL and the *TvHYP1* gene sequences, confirmed the presence of the transcript (Fig. 2B). The resulting PCR product was cloned and 3 out of 22 clones obtained by screening found to be undersized, 15 presented the expected size of approximately 1.5 kb, and 2 were longer than 1.5 kb. Two clones of 1.5 kb

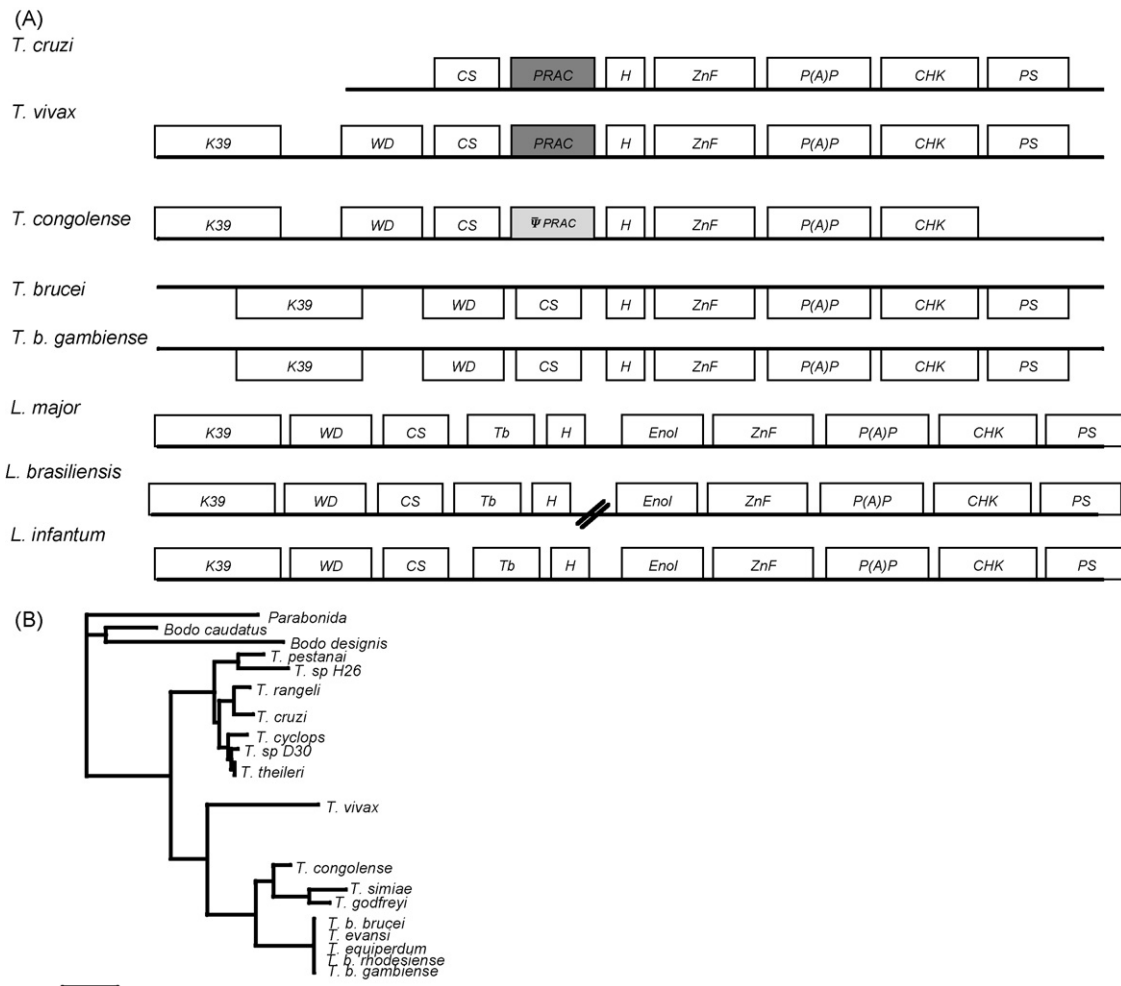


Fig. 1. PRAC locus analysis. (A) Assembled contigs were retrieved from GeneDB. CDS numbers are Tc00.1047053506795.80 (contig5391), Tc00.1047053509935.29 (contig 8250), tviv1929b09.p1k.8, congo1362b10.q1k.16, Tbgamb.14926, Tb927.7.3810, LM16BIN (contig2054), LbrM14.V2.1140 and LinJ14.1230 for *T. cruzi* (*TcPRACA* and *TcPRACB*), *T. vivax*, *T. congolense*, *T. brucei gambiense*, *T. b. brucei*, *Leishmania major*, *L. brasiliensis* and *L. infantum*, respectively. PRAC genes and pseudogene (Ψ) are shaded in grey. Flanking regions were annotated by GeneDB as the following: K39, kinesin 39; WD, WD domain containing protein; CS, cold shock domain containing protein; Tb, Tubby family protein; H, hypothetical protein; Enol, enolase; ZnF, zinc finger protein; P(A)P, poly(A) polymerase; CHK, carbohydrate kinase and PS, phosphatidyl serine synthetase. Parallel bars represent an additional region corresponding to *L. major* chromosome 34. (B) Phylogram based on SSU (V7–V8) rRNA gene sequences. Redrawn from Ref. [53]. Bar represents 0.1 substitutions per site.

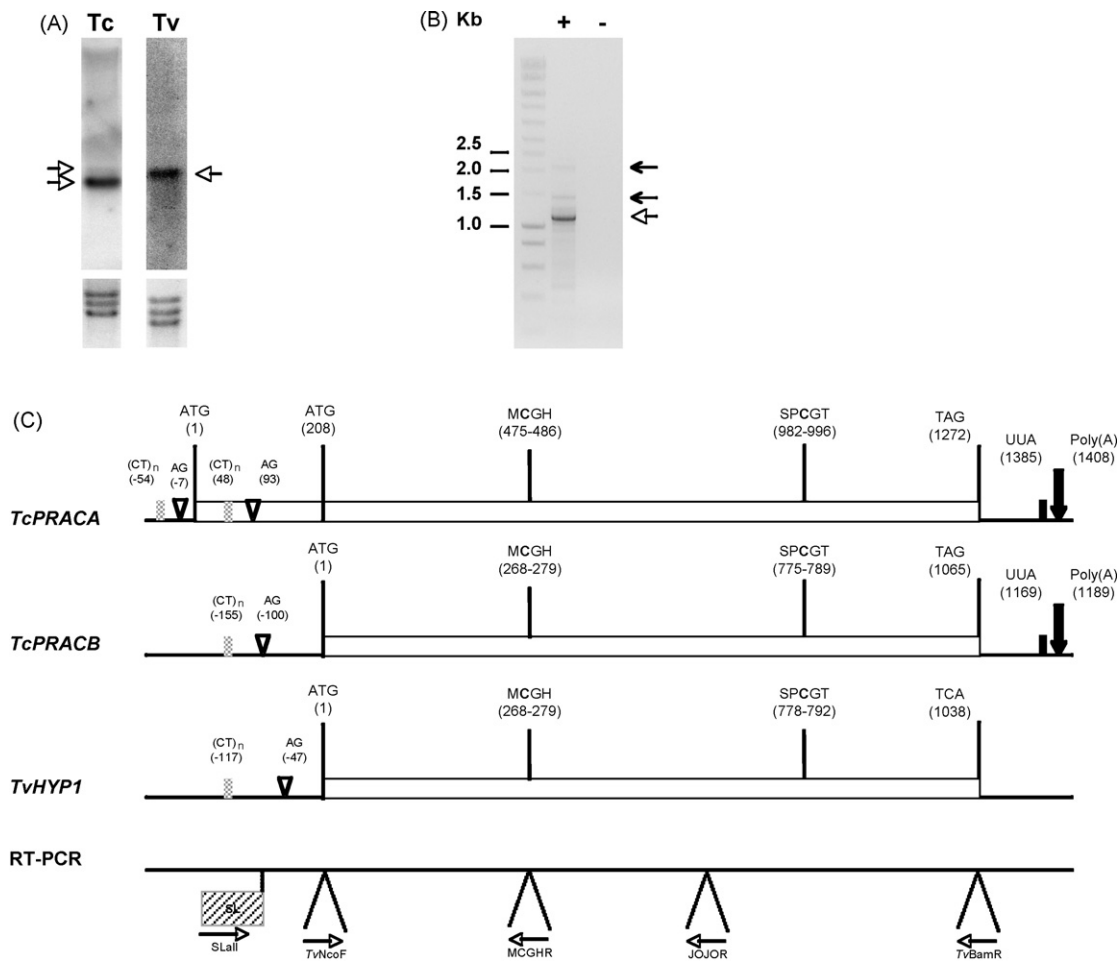


Fig. 2. Molecular characterization of *TvHYP1* expression. (A) Northern blot hybridization with *TcPRAC* or *TvHYP1* probes of total RNA from *T. cruzi* (*Tc*) or *T. vivax* (*Tv*) after separation on a 1.2% agarose/formaldehyde gel. Methylene blue staining of ribosomal RNA before hybridization is displayed below each lane. Arrows indicate two *TcPRAC* and one *TvHYP1* transcripts. (B) mRNA expression of *TvHYP1* from *T. vivax* blood stream forms shown by electrophoresis of the gene fragment obtained by oligo(dT)-primed reverse transcription from total RNA, followed by PCR amplification using the sequences of the mini-exon (spliced leader, SL) and *TvHYP1* specific primers. First strand cDNA reactions were performed in the presence (+) or absence (–) of reverse transcriptase to exclude the possibility of further PCR amplification of fragments due to genomic DNA contamination. (C) The full-length analysis of the *TvHYP1* is schematically represented as compared to *TcPRACA* and *TcPRACB* for references. It can be observed that the processing of parasite pre-mRNA occurred and led to monocistronic RNA presenting the acceptor site at position –47 of *TvHYP1*. The spliced leader acceptor sites polypyrimidine rich (CT) regions and start codons are indicated, as well as UUA triplets and polyadenylation sites. All sequences present the two characteristic PRAC motifs (MCGH and SPCGT) that encompass the two key catalytic residues. The figure shows the positions of all primers used throughout this study.

and two clones of the longer size were sequenced. While the longer clones led to irrelevant transcripts, both 1.5 kb clones encoded *TvHYP1* gene and allowed us to specifically locate the SL acceptor site. The schematic alignment of *TvHYP1* gene, with *TcPRACA* and *TcPRACB* gene sequences is presented in Fig. 2C. As it is shown on this figure, processing of *T. vivax* pre-mRNA had occurred and led to monocistronic RNA, with a single acceptor site at position –47 of *TvHYP1*. These results point out that *TvHYP1* sequence lacks a signal peptide and if transcribed would produce only an intracellular version of the enzyme. This is unlike the *TcPRACA* and *TcPRACB* genes that encode for secreted/membrane bound and/or intracellular proline racemases, respectively.

3.3. Analysis of PRAC activity of recombinant *TvHYP1*

Previous clarification of the catalytic mechanism of the dimeric *TcPRAC* [34] had demonstrated that the interconversion between L-proline and D-proline relies on two cysteine residues per enzyme subunit, namely Cys₁₃₀ and Cys₃₀₀. These key residues are build-in protein motifs, more specifically MCGH and MIII* (DRSPCGXGXAXXA) (see above and Ref. [25]). Recent studies revealed that these minimal patterns are not sufficiently stringent

to discriminate PRACs and hydroxyproline-2-epimerases (HypREs). These two enzymes are members of the PRAC family which possesses these protein motifs encompassing the two catalytic cysteines. However, three additional and non-dissociating sequence elements that are critical for substrate specificity (R1, R2 and R3) can be used to discriminate *in silico* PRAC from HypRE enzymes [26]. As can be seen in Fig. 3A, alignment of the *TvHYP1* amino acid sequence with *TcPRAC* reveals that the putative protein holds R1, R2 and R3 and both PRAC motifs that would be required to support proline racemase activity. These data are compatible with the 59% identity and 75% similarity observed with the *T. vivax* and *T. cruzi* sequences (Table 1).

To determine whether the product of the *TvHYP1* is a true proline racemase, the open reading frame of the *TvHYP1* gene obtained by PCR was cloned in pET28b(+) expression vector. The corresponding C-terminal His₆-tagged recombinant *TvHYP1* protein was produced in *E. coli* and purified by affinity chromatography using nickel-nitrilotriacetic acid–agarose columns. The r*TvHYP1* was purified to homogeneity, as revealed by specific anti-His antibodies (Fig. 3B) and it has an apparent molecular mass of 40 kDa. To test the ability of the soluble r*TvHYP1* protein to induce a shift in optical rotation of both L- and D-proline substrates, we used the previously

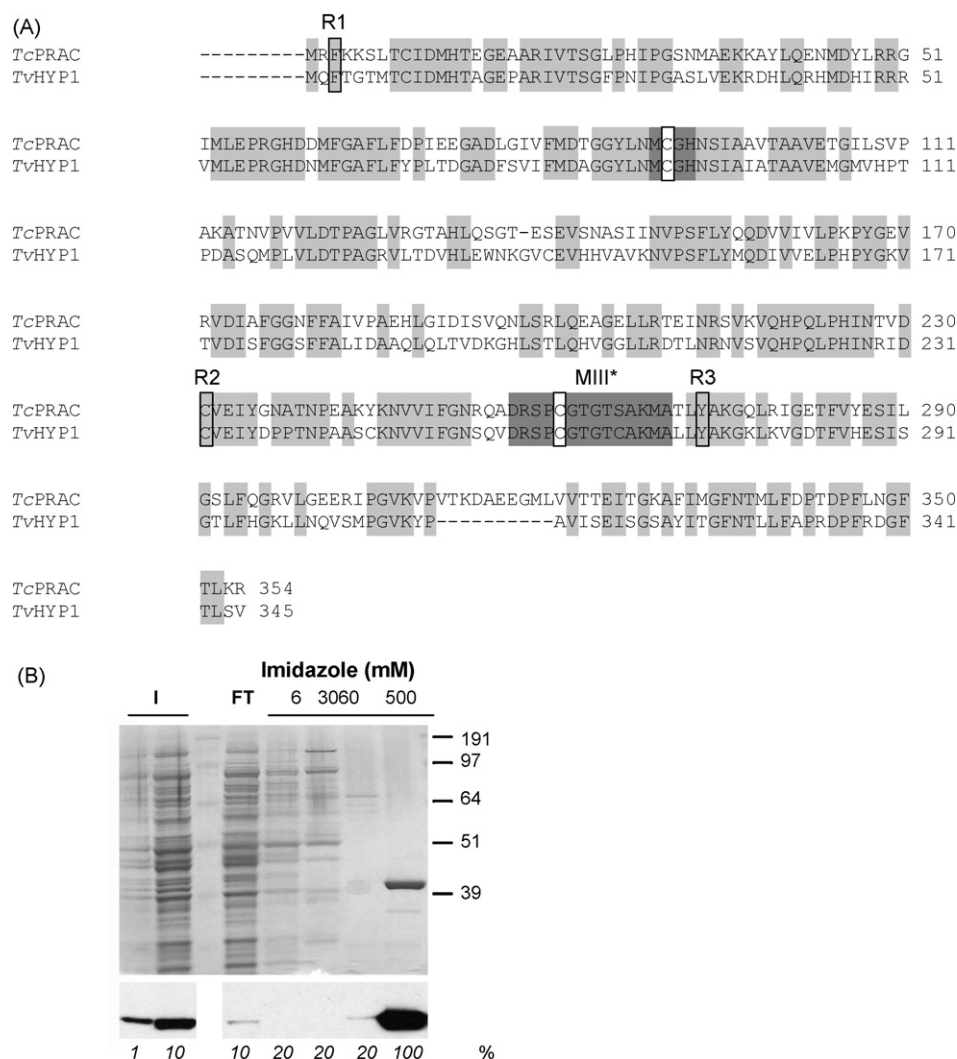


Fig. 3. *TvHYP1* sequence analysis and recombinant protein production. (A) Clustal W alignment of *TcPRAC* and *TvHYP1* protein sequences. Identical residues are shaded in light grey, MCGH and MIII* PRAC motifs are shaded in dark grey and catalytic cysteine residues are boxed. R1–R3 (boxed in black and shaded) indicate the critical differences allowing for the discrimination between PRAC and HyPRE. (B) *rTvHYP1* was purified from bacterial extracts using an IMAC column and eluted with increasing concentrations of imidazole (mM). Complexity of the different fractions was ascertained by 4–12% SDS-PAGE electrophoresis followed by Coomassie staining (upper panel) and specificity as well as efficient recovery was confirmed by Western blot (lower panel) using the anti-His Tag monoclonal antibody. Correspondences between the different amounts of loaded fractions are indicated below (%). I, input; FT, flow-through.

described proline racemase biochemical assays [23]. As it can be seen in Fig. 4A, *TvHYP1* protein racemizes both L- and D-proline but not hydroxy-L-proline or any other tested amino acid, corroborating the *in silico* analysis described above. Therefore, similarly to the parasite *TcPRAC* and to *Clostridium sticklandii* and *Clostridium difficile* PRACs, *TvHYP1* is a cofactor independent proline racemase [26,35]. This demonstrates that *TvHYP1* is *bona fide* a proline racemase, and it has been accordingly named as *TvPRAC*. In addition, no proline racemase activity was observed in soluble extracts obtained from available *T. congolense* and *T. brucei* cultured parasites, as compared to those prepared from *T. cruzi* epimastigotes (Fig. 4B). These data substantiate our findings in that the PRAC catalytic activity is not observed in trypanosoma species that lack PRAC genes.

The rate of conversion of L- into D-proline by recombinant *TvPRAC* calculated at various pH conditions clearly shows a pH dependency of the enzyme with an optimal *TvPRAC* activity at pH 6 (Fig. 4C). Furthermore, utilization of increasing amounts of PYC, the specific and competitive inhibitor of proline racemases, indicates that PYC equally inhibits *TvPRAC* enzymatic activity (Fig. 4D). To further determine the kinetic parameters of the enzyme, racemization assays were performed at 37 °C using *TvPRAC* optimum reaction

conditions. The recombinant *TvPRAC* exhibited Michaelis–Menten kinetics under the minimal substrate concentrations necessary for polarimetry. As depicted in Fig. 4E, analysis of L → D or D → L conversion of serial dilutions of proline catalyzed by identical amounts of *TvPRAC*, showed that the enzyme has a K_m of 145 and 106 mM and a V_{max} of 0.57 and $0.44 \times 10^{-4} \text{ mol s}^{-1}$ for L- and D-proline, respectively. To determine the apparent K_i value for PYC, kinetic assays were performed with 5 μM PYC and serial concentrations of 20–160 mM L-proline (Fig. 4F). These assays revealed that PYC is a competitive inhibitor of *TvPRAC*, as it is for *TcPRAC* and *CsPRAC*. The K_i value obtained for *TvPRAC* is 20 μM as compared to 6.7 and 18 μM for *TcPRAC* and *CsPRAC* enzymes, respectively (see Section 2).

3.4. *TvPRAC* is a conserved B-cell mitogen

The qualities of humoral and cellular responses triggered by parasites upon and during infectious processes are likely to be dependent on the degree of the microorganism confinement and also on mechanisms inherent to the immune system. Given that *T. vivax* and *T. congolense* were believed to be confined to the blood vessels, it was previously suggested that they belong to the “haematic”

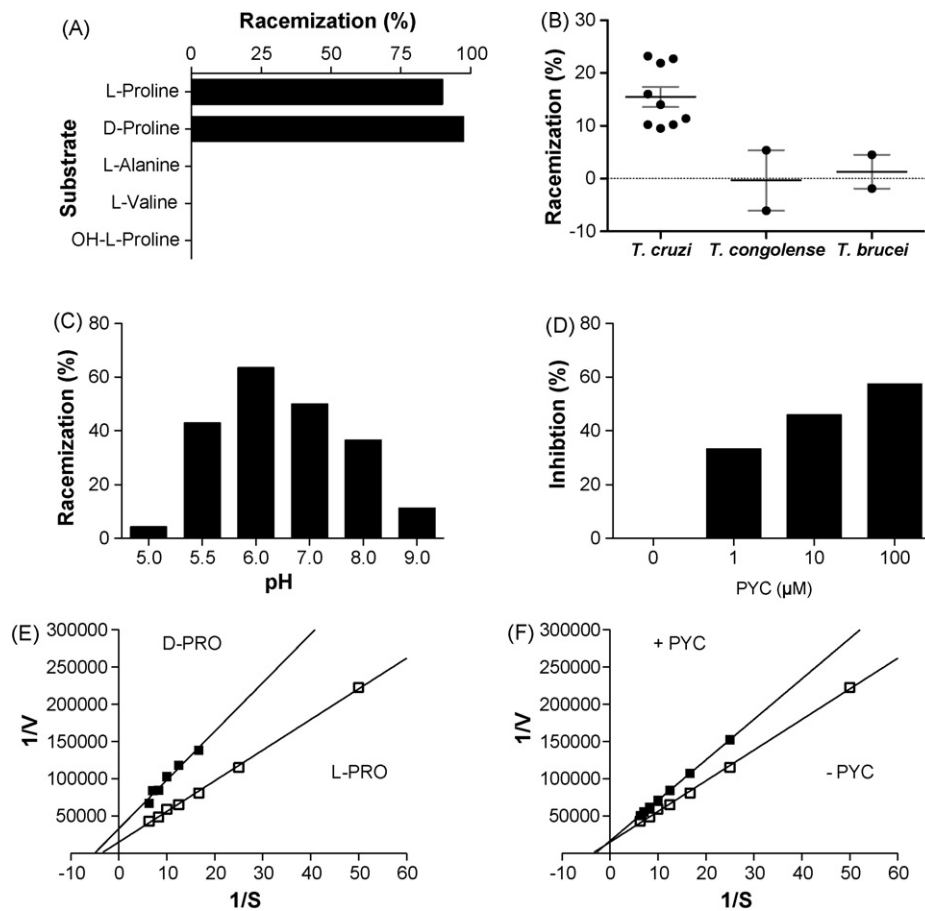


Fig. 4. Biochemical characterization of TvPRAC. Racemization assays were performed by incubating a 500 μ l mixture of 10 μ g of recombinant protein and 40 mM substrate in 0.2 M sodium acetate, for 30 min at 37 °C. The reaction was stopped by incubating at –20 °C. Water (1 ml) was then added and the optimal rotation measured in a polarimeter 241 MC as described in [23]. (A) Percent of racemization of 40 mM substrate, (B) percent of racemization of 20 mM L-proline, obtained with 50 μ g of soluble extracts from different culture forms of trypanosome species, each dot corresponding to individual experimental assays, (C) determination of optimum pH conditions for TvPRAC, and (D) percent inhibition of racemization of 40 mM L-proline induced by TvPRAC in the presence or in the absence of 1–100 μ M PYC. Initial rate of racemase activity was determined at 37 °C in medium containing 0.2 M sodium acetate pH 6.0, 20 μ g/ml of purified enzyme and different concentrations of L- or D-proline. (E) Lineweaver–Burk double reciprocal plots were used to determine values for K_m and V_{max} for L- (open squares) and D-proline (solid squares) where $1/V$ (V , initial speed, mol s^{-1}) is plotted in function of $1/S$ (S , substrate concentration, M), and the slope of the curve represents K_m/V_{max} . (F) Double reciprocal plot kinetics of 20 μ g/ml TvPRAC in the presence (solid squares) or absence (open squares) of 5 μ M PYC competitive inhibitor in function of 20–160 mM L-proline, where $1/V$ (V , initial speed, mol s^{-1}) is plotted in function of $1/S$ (S , substrate concentration, M). Graphic representation and linear curve regression allowed the determination of K_i as $[I]/[(\text{apparent } K_m/K_m) - 1]$.

group to which associated pathologies mostly result from the severe anaemia generated by the infection [36]. By contrast, *T. brucei brucei*, *T. b. rhodesiense* and *T. b. gambiense* were included in the “humoral” group responsible for high tissue inflammation and degeneration because they can be found both within vessels and extravascular tissue spaces. However, several studies have shown that *T. vivax* can also be found extravascularly [37]. Therefore *T. vivax* may affect the way the parasite interacts with cells of the host immune system, as well as the degree of immunosuppression and consequently the outcome of infection.

Attempting to test the ability of TvPRAC to stimulate B-lymphocytes and particularly whether it could contribute to the induction of “non-specific” hypergammaglobulinemia observed in acute phases of infection, *in vitro* proliferation assays were set up using naïve spleen cells from euthymic and athymic mice. We have found that rTvPRAC displays mitogenic activity towards splenic cells from euthymic Swiss mice, since addition of 100 μ g/ml of recombinant protein promotes a 13-fold increase of ^3H -thymidine incorporation when compared with untreated cells (Fig. 5A). A lymphoproliferative effect caused by contaminating *E. coli* endotoxine was ruled out since the levels of lymphocyte proliferation were not altered by the use of Polymyxine B (PMB) in the culture medium. Mitogenic activity of rTvPRAC seems to be dependent on the active

enzyme, since inhibition of rTvPRAC with 10 mM PYC prior to its incubation with splenocytes specifically decreased proliferation by 44%. This is in agreement with our previous results demonstrating that the mitogenic activity of proline racemases relies on the active (unliganded) conformation of the enzyme. Moreover, spleen cell proliferation induced by rTvPRAC increased with time over 72 h of culture (Fig. 5B). A dose-dependent with a bell-shaped curve response (starting at 0.8 μ g/ml and peaking at 100 μ g/ml), has been shown, typical to all B-cell mitogens described so far (Fig. 5B). Specific cultures were then seeded using splenic cells from athymic Swiss (nu⁻/nu⁻) mice (Fig. 5C). Higher levels of cell proliferation were obtained in absence of T-cells providing evidence that rTvPRAC is a T-cell-independent B-cell mitogen. These data are consistent with an exclusive B-cell composition of the cell suspensions. Therefore they clarify the partial reduction of lymphoproliferation observed when total (B- and T-) spleen cell suspensions from euthymic mice were incubated with previously inactivated enzyme. To further evaluate the impact of TvPRAC *in vivo*, the recombinant protein was injected to naïve mice and B-cell responses have been monitored at several time points after injection. As seen in Table 2, while B-cell numbers increase twofold in the first week of infection, a 3–20-fold increase of secreting B-cells was observed, depending on the immunoglobulin class and isotype. Together with the

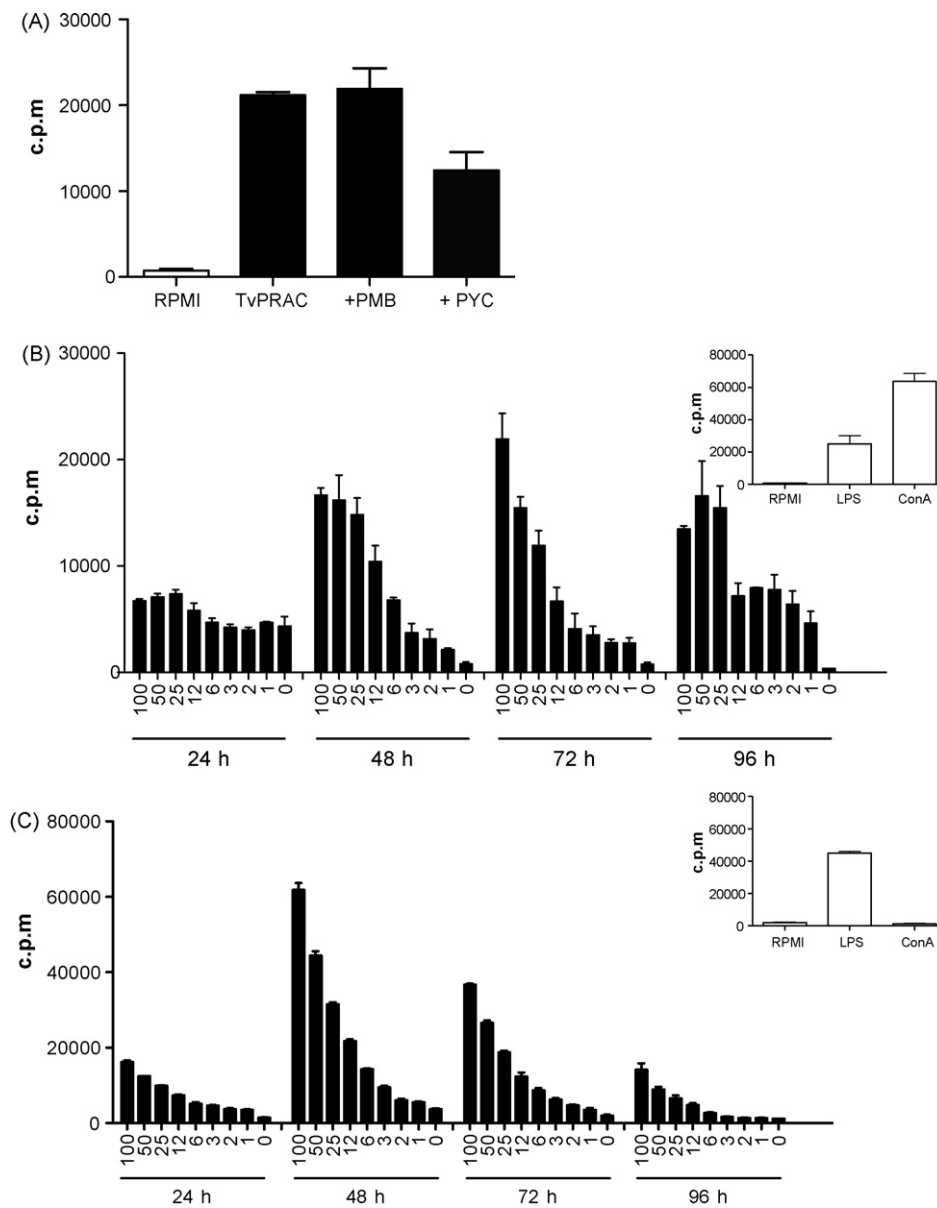


Fig. 5. Characterization of rTvPRAC mitogenic activity. (A) Proliferative activity (c.p.m., counts/min of ^3H -thymidine) of total splenocytes 72 h post-incubation with recombinant 50 $\mu\text{g/ml}$ of rTvPRAC in the presence of Polymyxine B (PMB) or after incubation with pyrrole-2-carboxylic acid (PYC). (B) Proliferative activity (c.p.m.) of total splenocytes from Swiss mice in the presence of increasing concentrations (below graph) of rTvPRAC and 2.5 $\mu\text{g/ml}$ of PMB. (C) Proliferative activity of total splenocytes from Swiss nu^-/nu^- mice in the presence of increasing concentrations (below graph) of rTvPRAC and 2.5 $\mu\text{g/ml}$ of PMB. Insets, ^3H -thymidine uptake (c.p.m.) after 72 h in the presence of RPMI medium, lipopolysaccharide (LPS) or concavalin A (ConA).

Table 2
In vivo mitogenic activity of TvPRAC.

	Day 0	Day 2	Day 5	Day 10
Spleen cells $\times 10^8$	0.97 \pm 0.12	1.03 \pm 0.18	1.39 \pm 0.05	1.80 \pm 0.25
IgM secreting cells $\times 10^4$	32.1 \pm 17.6	76.0 \pm 6.1	384.0 \pm 40.9	57.4 \pm 0.0
IgG secreting cells $\times 10^4$	35.8 \pm 13.5	26.4 \pm 6.0	70.1 \pm 1.3	33.1 \pm 5.6
IgG1 secreting cells $\times 10^4$	12.5 \pm 5.3	17.9 \pm 2.0	75.7 \pm 3.2	ND
IgG2a secreting cells $\times 10^4$	6.4 \pm 1.1	13.4 \pm 0.5	106.4 \pm 14.9	11.6 \pm 6.1
IgG2b secreting cells $\times 10^4$	22.7 \pm 6.3	18.4 \pm 0.0	30.6 \pm 8.1	16.7 \pm 8.3
IgG3 secreting cells $\times 10^4$	11.5 \pm 2.4	12.1 \pm 2.7	70.9 \pm 7.8	25.3 \pm 16.7
IgM (mg/ml)	0.06 \pm 0.01	0.08 \pm 0.02	0.15 \pm 0.09	0.09 \pm 0.02
IgG (mg/ml)	1.9 \pm 0.6	1.9 \pm 1.1	3.7 \pm 1.2	3.1 \pm 0.6
Titer TvPRAC				
IgM	1.0 \pm 0.0	1.0 \pm 0.0	130.0 \pm 28.3	216.7 \pm 76.4
IgG	1.0 \pm 0.0	1.0 \pm 0.0	120.0 \pm 28.3	700.0 \pm 141.4

high titers of antibodies observed in the mouse sera, the present results suggest that rTvPRAC triggers high levels of B-cell activation, terminal differentiation and antibody secretion.

4. Discussion

Previous studies have ascribed a particular importance in parasite development and acquisition of virulence to TcPRAC [24]. Despite the significant degree of gene synteny presented by genomes of the *Trypanosomatidae* family members and the high conservation of the PRAC locus environment, the present work provided evidence that only *T. vivax* genome displays one orthologue of TcPRAC. Moreover, the results revealed that the gene encodes for a functional enzyme – TvPRAC – with molecular and biochemical characteristics equivalent to TcPRAC. Similarly to *T. cruzi* and other bacterial PRACs, TvPRAC is also a T-cell-independent B-cell mitogen.

The ability of *T. vivax* and *T. cruzi* to produce functional PRACs, in contrast to other closely related kinetoplastids, is puzzling. However, attention is drawn to the fact that genes coding for essential proteins associated to key metabolic reactions are not necessarily ubiquitous among members of the same family. In this context, it is interesting to recall that many enzymes involved in energy resources are not invariably present in all trypanosomes and that various alternative, species- or stage-specific, biochemical pathways are build-in to provide a carbon supply [8,38]. It is widely accepted that morphological and biochemical changes occurring in the course of the biological cycle of trypanosomatids rely on parasite adaptation to more or less complex host environments [39–41] and to their ability to make intracellular reserve of energy [42]. Accordingly, metabolic requirements will be directly related to the stage of the parasite development in extracellular or intracellular compartments, i.e. in mammalian bloodstream, within host cells and tissues or in the lumen of the insect gut. Since glucose concentration vary considerably in these different environments thus affecting the energy supply, a switch to alternative carbon sources by trypanosomes is conditioned by the glucose availability in the milieu [7–8]. However, identification of one or more proline transporter systems at the membrane of several trypanosomatids, notably of *T. cruzi*, had assigned to proline a significant role in parasite metabolism, in presence or absence of glucose [18–20]. Accordingly, although no formal evidence is available for the presence of specific proline transporters in *T. vivax*, successful axenic parasite cultures seem to be influenced by the L-proline concentration in the medium [43]. Therefore, it cannot be ruled out, at present, that *T. vivax* development is dependent on the levels of L-proline uptake and consequently on its intracellular availability. While the clear function of TvPRAC still remains poorly understood, we suggest that the enzyme may well interfere with parasite metabolic reactions by the regulation of the cytoplasmic pool of L-/D-proline, as suggested for *T. cruzi* [25].

Similarly, the fact that trypanosomatids possess different energy requirements and carry distinct abilities to breakdown amino acids may also explain the absence of PRAC genes in *T. brucei*, *T. congolense* and *Leishmania* spp. For example, in addition to proline, *T. cruzi* can extensively benefit from histidine and serine catabolism, in contrast to *T. brucei* and *T. congolense* [31,39,44,45]. In turn, promastigote and amastigote forms of *Leishmania* spp. alternatively use particular sets of enzymes to process disaccharides or fatty acids [9]. Furthermore, *Leishmania amazonensis* takes major metabolic advantages from the alanine metabolism since the D-alanine available in the parasite cytoplasm through an alanine racemase-dependent process is used in the peptidoglycan biosynthesis [12]. It is interesting to note that alanine represents more than 30% of the intracellular pool of free amino acids in *Leishmania tropica* [46] and together with alanine racemase constitutes a potent regulator of osmotic stress resulting from variations in environmental tonicity [47–49].

Because *T. vivax* and *T. cruzi* developmental biology and fate are substantially different, both during the insect and the mammalian stages, straightforward correspondences cannot be established, justifying the presence of functional PRACs in these trypanosomes. Additional difficulties are imposed by the different gene expression and regulation observed in these parasites, as well as by distinct localization of homologous proteins in intracellular and/or extracellular parasite/host spaces. For instance, the genomic organization and transcription of TvPRAC suggests that only one copy of the gene is present per haploid genome and encodes a protein lacking a signal peptide. In contrast, *T. cruzi* possesses at least two copies of the PRAC gene, which encode for endogenous and/or exogenous TcPRAC isoforms, seemingly playing distinct roles during development according to the extent of parasite differentiation and its localization. Finally, taking into consideration the fact that *T. vivax* multiplies extracellularly in the host bloodstream and given that TvPRAC possesses mitogenic activity to host B-cells, it is conceivable that the protein would take part in triggering non-specific polyclonal B-cell responses. Therefore the resulting characteristic host immunosuppression would contribute to the parasite evasion and persistence. Since TvPRAC sequence does not possess a signal peptide for secretion, it is tempting to assume that the protein would reach the bloodstream and/or extravascular spaces through the parasite flagellar pocket, in which intense exocytic/endocytic activities take place. It seems plausible as well that TvPRAC would be accessible for B-cells as a result of the significant destruction of the parasites. This could involve innate and/or acquired host mechanisms, further contributing to the maintenance of B-cell activation and to the increase in antibody diversity. Ongoing experiments are underway to verify these hypotheses and further examine the biological role of PRAC in *T. vivax*.

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