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

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ABSTRACT

Trypanosoma cruzi proline racemases (*Tc*PRAC) are the only eukaryotic proline racemases described so far. Except their role in the interconversion of free L- and D-proline enantiomers, parasite *Tc*PRACs 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 *Tc*PRAC. Inhibition studies using the pyrrole-2-carboxylic acid confirmed that this compound is a competitive inhibitor for both *Tc*PRAC and *Tv*PRAC enzymes. Similarly to *Tc*PRAC and all members of the racemase family studied so far in other pathogenic and nosocomial bacteria, our results show that *Tv*PRAC 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-born 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* (*Tc*PRAC) that is differentially expressed in all stages of *T. cruzi*

☆ *Note:* Nucleotide sequence data reported in this paper for *Trypanosoma vivax* proline racemase (*Tv*PRAC) are available in the GenBank^{TM}, under the accession number EF175213.

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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 *Tv*PRAC 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 *Tc*PRAC enzymes. We have also demonstrated both *in vitro* and *in vivo* that *Tv*PRAC 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 sonication 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 *Tv*NcoF forward (5′-GGCGCCATGGAGTTCACCGGAACAATG-3′) and *Tv*BamR reverse (5′-CCGGGATCCACGCTCAGCGTAAAGCC-3′) primers and then labelled with [α-^{32}P]-dCTP using megaprime DNA labeling system (Amersham Life Science). Blots were hybridized overnight in ULTRAhyb^{TM} 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: *Tv*SL: 5′-AGAACAGTTTCTGTACTATATTG-3′, SL-all: 5′-TRRWACAGTTTCTGTACTATATTG-3′, *Tv*BamR 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 *Tv*NcoF and *Tv*BamR primers, and cloned into NcoI/BamHI sites of pET28b(+) expression vector (Novagen/Merck) using the Rapid Ligation Kit (Roche). *Escherichia coli* DH5α 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

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