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Trypanosoma cruzi proline racemases are involved in parasite differentiation and infectivity

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Summary

Polyclonal lymphocyte activation is one of the major immunological disturbances observed after microbial infections and among the primary strategies used by the parasite *Trypanosoma cruzi* to avoid specific immune responses and ensure survival. *T. cruzi* is the insect-transmitted protozoan responsible for Chagas' disease, the third public health problem in Latin America. During infection of its mammalian host, the parasite secretes a proline racemase that contributes to parasite immune evasion by acting as a B-cell mitogen. This enzyme is the first described eukaryotic amino acid racemase and is encoded by two paralogous genes per parasite haploid genome, *TcPRACA* and *TcPRACB* that give rise, respectively, to secreted and intracellular protein isoforms. While *TcPRACB* encodes an intracellular enzyme, analysis of *TcPRACA* paralogue revealed putative signals allowing the generation of an additional, non-secreted isoform of proline racemase by an alternative *trans*-splicing mechanism. Here, we demonstrate that overexpression of *Tc*PRAC leads to an increase in parasite differentiation into infective forms and in its subsequent penetration into host cells. Furthermore, a critical impairment of parasite viability was observed in functional knock-down parasites. These results strongly emphasize that *Tc*PRAC is a potential target for drug design as well as for immunomodulation of parasite-induced B-cell polyclonal activation.

Introduction

Chagas' disease is a complex tissue pathology induced by *Trypanosoma cruzi* and is endemic in Latin America. Effective therapies against this parasite infection are restricted to the short acute phase of the disease and rely on only two compounds. Some patients do not respond to the available treatment and cure is subjected to several criteria, such as susceptibility of the parasite strain, age of the host and stage of the disease. The ability of *T. cruzi* to induce several host immune system dysfunctions makes the development of effective vaccines and of new treatments a difficult task.

Trypanosoma cruzi is a digenetic parasite that develops through a complex life cycle involving an insect vector and a mammalian host where the various different parasite developmental stages differ in their multiplication and infectious abilities as well as in their metabolic requirements. For instance, proline is an important source of energy for *T. cruzi* (Sylvester and Krassner, 1976) and efficient L-proline uptake by the parasite in those widely different environments (Silber *et al*., 2002) is guaranteed by high- or low-affinity active proline transporter systems. Furthermore, proline is an essential effector molecule for the signalling cascade that initiates attachment to the rectal cuticle by hindgut non-infective epimastigotes as the initial step towards differentiation into metacyclic non-replicative forms that are infectious to mammals (Kollien and Schaub, 2000). Whereas acidity, osmolarity, blood degradation products and stress are some of the factors influencing metacyclogenesis, appreciable concentrations of free proline present in the insect urine may also influence parasite differentiation (Contreras *et al*., 1985; Kollien *et al*., 2001). Therefore, the precise mechanisms involved in the development of epimastigote stages and the acquisition of infectivity by metacyclic forms have not yet been elucidated. The finding of proline racemase (PR) genes in *T. cruzi* has strengthened the notion that proline plays an essential role in the parasite metabolism (Reina-San-Martin *et al*., 2000; Chamond *et al*., 2003).

Proteins are essentially composed of L-amino acids; however, an increasing number of reports have described the intimate relationship between free or protein-bound D-amino acids and toxins (Cheng and Walton, 2000), breakdown-resistant cell wall structures (Heaton *et al*., 1988; Thompson *et al*., 1998), malignancies and progressive disorders (Nagata *et al*., 1987; Fisher, 1998; Silbernagl *et al*., 1999). D-amino acids can be used as a source of carbon in some bacteria (Janes and Bender, 1999) and

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might act as regulator of their L- counterpart pool. However, most of the reports deal with the fact that both Gram-positive and Gram-negative bacteria have D-alanine and D-glutamate incorporated in their cell wall structures (see Walsh, 1989, for review), and contain racemases to produce the necessary D-enantiomer. In pathogenic bacteria, the genetic inactivation of amino acid racemase genes abolishes bacterial pathogenicity by the consequent inability to synthesize the D-amino acid-containing mucopeptide of the bacterial cell wall, a necessary component protecting bacteria from the external milieu (Thompson *et al*., 1998).

Trypanosoma cruzi PR are pyridoxal independent enzymes that interconvert free L- and D-proline enantiomers (Reina-San-Martin *et al*., 2000) and are encoded in the parasite by two paralogous genes per haploid genome, namely *TcPRACA* and *TcPRACB*. Both genes encode enzymes with 96% identical amino acid sequences but with distinct kinetic properties that may be relevant for their catalytic activities (Chamond *et al*., 2003). Moreover, two protein isoforms of PR differentially expressed during *T. cruzi* development have been described. A PR of monomer size of 45 kDa (*Tc*PRACA) is only expressed and released by infective metacyclic and bloodstream forms of the parasite. Another protein isoform with an intracellular localization and with a monomer size of 39 kDa is mainly expressed in non-infective epimastigote forms of *T. cruzi*. The secreted isoform *Tc*PRACA is an efficient mitogen for host B lymphocytes and is significantly implicated in non-specific polyclonal activation of lymphoid cells preventing the development of effective parasite-directed immune responses. It has been suggested that the cytoplasmic PR isoform plays a role in the regulation of intracellular metabolic pathways in *T. cruzi* epimastigote forms (Reina-San-Martin *et al*., 2000; Chamond *et al*., 2003).

Genomic studies on the organization and on the transcription of *TcPRAC* genes revealed that the *TcPRACA* coding sequence, contrasting to that of *TcPRACB*, encompasses an extra hydrophobic N-terminal domain, consisting of a signal which ensures secretion of the secreted isoform of PR. However, *TcPRACA* also possesses two alternative *trans*-splicing signals and appropriate downstream spliced-leader acceptor sites followed by alternative translation initiation codons (Reina-San-Martin *et al*., 2000; Chamond *et al*., 2003). These features would theoretically enable the generation of a putative intracellular enzyme as well as the extracellular enzyme from the same racemase gene. Although functional intracellular PR can indeed be encoded by the *TcPRACB* gene (Chamond *et al*., 2003), the ability of *TcPRACA* to express an intracellular PR form by alternative *trans*-splicing remains to be proven.

Here, we first confirmed that alternative *trans*-splicing of *TcPRACA* may indeed occur in epimastigotes. Through this mechanism, mRNAs encoding the intracellular PR isoform are produced at this parasite stage from *Tc*PRACA and *Tc*PRACB genes. Second, we generated transgenic parasites for *TcPRAC* genes and analysed the ability of mutants to pursue metacyclogenesis and to interact with host cells. The data we obtained determined that overexpression, but not reduction, of *TcPRAC* genes in non-infective forms leads to an increase in parasite differentiation *in vitro* and enhanced parasite infectivity.

Results

Trans-*splicing of* TcPRACA *transcript and production of a version of proline racemase that lacks a signal for secretion*

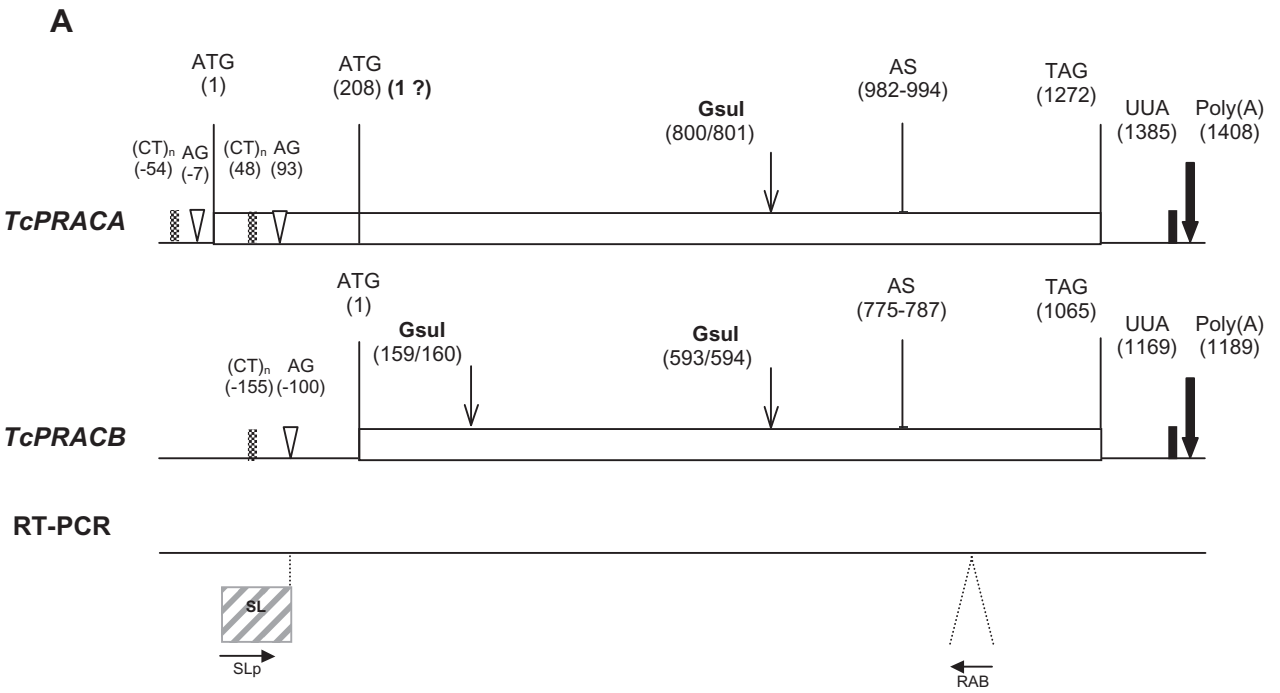
Previous genomic sequence analysis of PR genes and their flanking regions predicted that *TcPRACA* gene could generate both secreted and intracellular isoforms of PR (Reina-San-Martin *et al*., 2000). *TcPRACA* has two polypyrimidine-rich motifs (positions –54 and +48) that are essential trypanosomatid *trans*-splicing signals when located upstream of an (AG)-dinucleotide used as an acceptor site for the spliced leader (Fig. 1A). As for other trypanosome genes, UUA nucleotide triplets precede the polyadenylation site located downstream of the coding region. Compared with the *TcPRACB* paralogue, whose sequence shares 96% identity, the longer transcript derived from *TcPRACA* encodes an extra 5′ domain leading to an open reading frame of 423 codons possessing an export domain for the secreted isoform of PR. Furthermore, comparison of *TcPRACA* (+208 to +1272) and *TcPRACB* (+1 to +1065) sequences indicated the presence of 14 point mutations, one of which leading to a differential GsuI site (Chamond *et al*., 2003). DNA from recombinant genomic lambda phages specific for each gene sequence (Reina-San-Martin *et al*., 2000) was polymerase chain reaction (PCR) amplified with *TcPRACA* and *TcPRACB* common primers and a characteristic restriction site profile was observed for each gene when the GsuI enzyme was applied (Fig. 1B). The resulting restriction patterns can easily differentiate cloned *TcPRACA* or *TcPRACB* genes while GsuI restriction of PCR product obtained from genomic DNA of CL strain parasites had the predicted mixed profile (Fig. 1B, first lane). Transcriptional analysis was next performed using oligo(dT)-primed reverse transcription of total RNA from different parasite life stages and nested PCR amplifications using a set of primers specific for the spliced leader (SLp) or RAB common reverse sequences (see Fig. 1A) further confirmed the presence of the respective *TcPRAC* transcripts throughout parasite development (Fig. 1C). GsuI treatment of reverse transcribed polymerase chain

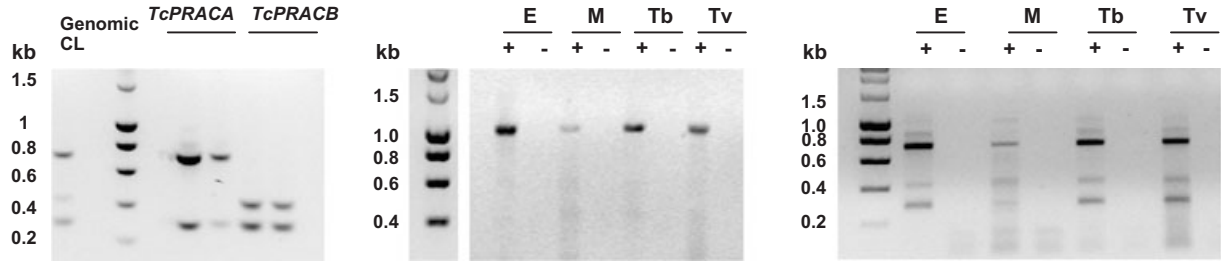
reaction (RT-PCR) fragments gave a mixed digestion profile indicating that both *TcPRACA* and *TcPRACB* genes are transcribed in all the parasite life stages analysed (Fig. 1D). While these findings agree with the presence of both intracellular and secreted versions of the protein in metacyclic, bloodstream forms and trypomastigotes obtained from cultures, as previously demonstrated by immunofluorescence analysis and peptide sequencing (Reina-San-Martin *et al*., 2000), it was intriguing to envisage that epimastigote forms that are not able to secrete the *Tc*PRACA PR isoform (Reina-San-Martin *et al*., 2000; Chamond *et al*., 2003) would express both *TcPRACA* and *TcPRACB* transcripts. This raised the possibility that the second initiation codon (position +208) observed in *TcPRACA* sequence could also be used by alternative *trans*-splicing. To verify this hypothesis SLp/RAB-amplified fragments obtained by reverse transcription of epimastigote RNA were cloned and sequenced. Analysis of the resulting sequences demonstrated the exact positioning of SL on acceptor sites (positions +93 and −100) of the, respectively, *TcPRACA* and *TcPRACB* sequences (Fig. 1E). The respective point mutations characteristic of each of the paralogous genes leading to defined GsuI restriction sites in the respective sequences were observed. Although this confirms the expression of *TcPRACB*, it also unambiguously highlights that *TcPRACA* can generate *in vivo* a putative intracellular version of the protein by alternative splicing.

Overexpression of Tc*PRAC proline racemase genes in* T. cruzi *and characterization of transfected parasites*

To appreciate the relative biological relevance of *T. cruzi* PR, *TcPRACA* and *TcPRACB* paralogues encoding, respectively, the secreted and intracellular versions of the protein were overexpressed in non-infective but replicative epimastigote forms of the parasite by stable chromosomal integration using the pTREX-n vector (Vazquez and Levin, 1999). pTREX-n is characterized by its ability to integrate more efficiently into the *T. cruzi* genome (see below) and to favour the creation of stably transfected parasites. pTREX-n plasmid, a derivative of pRIBOTEX (Martinez-Calvillo *et al*., 1997), retains a *neo* gene as a positive selectable marker and possesses a region HX1 immediately after the ribosomal promoter that provides a more efficient *trans*-splicing signal than the cryptic ones described previously and directs rapid expression of genes inserted downstream of it. We overexpressed full-length *TcPRACA* and *TcPRACB* genes cloned in sense or antisense orientations to obtain, respectively, transfectants overexpressing *Tc*PRACA and *Tc*PRACB, or potentially functional knock-down parasites for natural *Tc*PRAC. Transfected parasites were selected using two drug concentrations (200 and 500 $\mu g\ ml^{-1}$) in an attempt to select for low- and high-level expressors, and were further cloned by limiting dilution. Interestingly, parasites transfected with pTREX-*Tc*PRACA vectors, either in the sense or anti-

Fig. 1. RT-PCR analysis of *TcPRACA* and *TcPRACB* gene expression during *T. cruzi* development.
A. The schematic representation of genomic organization of *TcPRACA* and *TcPRACB* is depicted by a common nucleotide scale along the linear alignment of both sequences where the main positions are indicated in parentheses. Spliced leader acceptor sites (AG) are represented as open triangles and positions of characteristic *trans*-splicing polypyrimidine rich-signal domains $(CT)_n$, UUA nucleotide triplets and polyadenylation sites [Poly(A)] are indicated, as well as the conserved active site (AS) sequences for both enzyme isoforms. *TcPRACA* sequence presents an (AG) acceptor site at position (–7) allowing for the production of the secreted isoform of PR whose translation of a signal peptide domain starts at position (1) (Reina-San-Martin *et al*., 2000) and an alternative (AG) acceptor site at position (+93) allowing for the putative initiation codon of an intracellular isoform of PR starting at position (+208). This contrasts with *TcPRACB* sequence, that although presenting 96% identity was shown to simply encode a truncated intracellular protein with a start codon (position 1) homologous to the second ATG of *TcPRACA* gene. Predicted restriction sites for GsuI enzyme are indicated for both *TcPRACA* and *TcPRACB*. Spliced leader (SLp) and RAB common reverse oligonucleotides are indicated to illustrate the reverse transcription strategy used.
B. The digestion of (Hi/RAB) fragments obtained by PCR of DNA from original individual phages containing *TcPRACA* or *TcPRACB* genes (Reina-San-Martin *et al*., 2000) revealed that *TcPRACA* or *TcPRACB* can be differentiated by GsuI restriction site analysis and that a mixed profile was obtained when using parasite genomic DNA (Genomic CL) as template.
C. The presence of PR transcripts during parasite development was ascertained by oligo(dT)-primed reverse transcription of total parasite RNA followed by PCR amplification using the sequences of the mini-exon (spliced leader, SLp) and RAB 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. Gel electrophoresis of corresponding amplified fragments from E, epimastigote, M, purified metacyclic, Tb, blood trypomastigote, and Tv, cultured trypomastigote parasite forms, is shown.
D. Further GsuI digestion of RT-PCR-amplified transcripts from the different parasite forms revealed both *TcPRACA* and *TcPRACB* gene restriction profiles, indicating that both genes are transcribed during all parasite stages of development. Molecular weights are indicated in margins.
E. The RT-PCR analysis of cloned SLp/RAB amplified fragments obtained from epimastigote RNA were compared by sequencing and showed that processing of parasite pre-mRNA occurred and led to monocistronic RNA presenting the spliced leader positioned in the alternative acceptor site at position (+93) of *TcPRACA* gene or in the position (–100) of *TcPRACB* gene. These sequences (seq 1 and seq 2) which are representative of several different experiments confirmed that both *TcPRACA* and *TcPRACB* genes encode the intracellular isoform of PR at this stage of parasite development as ascertained by the respective synonymous mutations characteristic of *TcPRACA* or *TcPRACB* genes (in light and dark grey respectively), or by non-synonymous mutations leading to amino acid changes in *Tc*PRACA and *Tc*PRACB PR (light and dark green respectively), and by corresponding characteristic GsuI restriction sites (are highlighted in red). Non-coding sequences are shown in italic; spliced leader acceptor sites are underlined in bold, and their positions in the sequences are indicated; translation initiation codons are highlighted in yellow. Primers used for PCR amplification are indicated in a red font. For clarity, not full-length *TcPRACA* and *TcPRACB* sequences are shown.





E

(+93)

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TcPRACA     1 ---CCCATTTTTTTTTTTTTTTTGTGTGTTT-CCCTTGATCTCTCGAACAGGCAGGAAAAGCTTCTGTTTGACCAAAAATATAAAATTATTAAGGGCGA 96
Seq 1       1 ----------------AACGCTATTATTGATACAGTTTCTGTACTATATTGGGCAGGAAAAGCTTCTGTTTGACCAAAAATATAAAATTATTAAGGGCGA 84
Seq 2       1 --------------------AACGCTATTATTGATACAGTTTCTGTACTATATTGGAAAAGCTTCTGTTTGACCAAAAATATAAAATTATTAAGGACGA 79
TcPRACB     1 CCCAACATTTTTTTTTTTTTTTTGTGTGTTTTCCCTTGATTCC-CGAACGGGGCAGGAAAAGCTTCTGTTTGACCAAAAATATAAAATTATTAAGGACGA 99
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(-100)

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TcPRACA    97 GAAAAAAGAAAAGAAAAAAAATCAACGAGCAAACAGGAGAGAACAACAACAA--AAAAGGGAAATTATGCGATTTAAGAAATCATTCACATGCATCGACA 194
Seq 1      85 GAAAAAAGAAAAGAAAAAAAATCAACGAGCAAACAGGAGAGAACAACAACAA--AAAAGGGAAATTATGCGATTTAAGAAATCATTCACATGCATCGACA 182
Seq 2      80 GAAAA--GAAA---AAAAAAATCAAACC-------GGAGAGAACAACAACAACAAAAAGGGAAATTATGCGATTTAAGAAATCATTCACATGCATCGACA 167
TcPRACB   100 GAAAA--GAAA---AAAAAAATCAAACC-------GGAGAGAACAACAACAACAAAAAGGGAAATTATGCGATTTAAGAAATCATTCACATGCATCGACA 187

TcPRACA   195 TGCATACGGAAGGTGAAGCAGCACGGATTGTGACGAGTGGTTTGCCACACATTCCAGGTTCGAATATGGCGGAGAAGAAAGCATACCTGCAGGAAAACAT 294
Seq 1     183 TGCATACGGAAGGTGAAGCAGCACGGATTGTGACGAGTGGTTTGCCACACATTCCAGGTTCGAATATGGCGGAGAAGAAAGCATACCTGCAGGAAAACAT 282
Seq 2     168 TGCATACGGAAGGTGAAGCAGCACGGATTGTGACGAGTGGTTTGCCACACATTCCAGGTTCGAATATGGCGGAGAAGAAAGCATACCTGCAGGAAAACAT 267
TcPRACB   188 TGCATACGGAAGGTGAAGCAGCACGGATTGTGACGAGTGGTTTGCCACACATTCCAGGTTCGAATATGGCGGAGAAGAAAGCATACCTGCAGGAAAACAT 287

TcPRACA   295 GGATTATTTGAGGCGTGGCATAATGCTGGAACCACGTGGTCATGATGATATGTTTGGAGCCTTTTTATTTGACCCTATTGAAGAAGGCGCTGACTTGGGC 394
Seq 1     283 GGATTATTTGAGGCGTGGCATAATGCTGGAACCACGTGGTCATGATGATATGTTTGGAGCCTTTTTATTTGACCCTATTGAAGAAGGCGCTGACTTGGGC 382
Seq 2     268 GGATTATTTGAGGCGTGGCATAATGCTGGAGCCACGTGGTCATGATGATATGTTTGGAGCCTTTTTATTTGACCCTATTGAAGAAGGCGCTGACTTGGGC 367
TcPRACB   288 GGATTATTTGAGGCGTGGCATAATGCTGGAGCCACGTGGTCATGATGATATGTTTGGAGCCTTTTTATTTGACCCTATTGAAGAAGGCGCTGACTTGGGC 387
                                      GsuI
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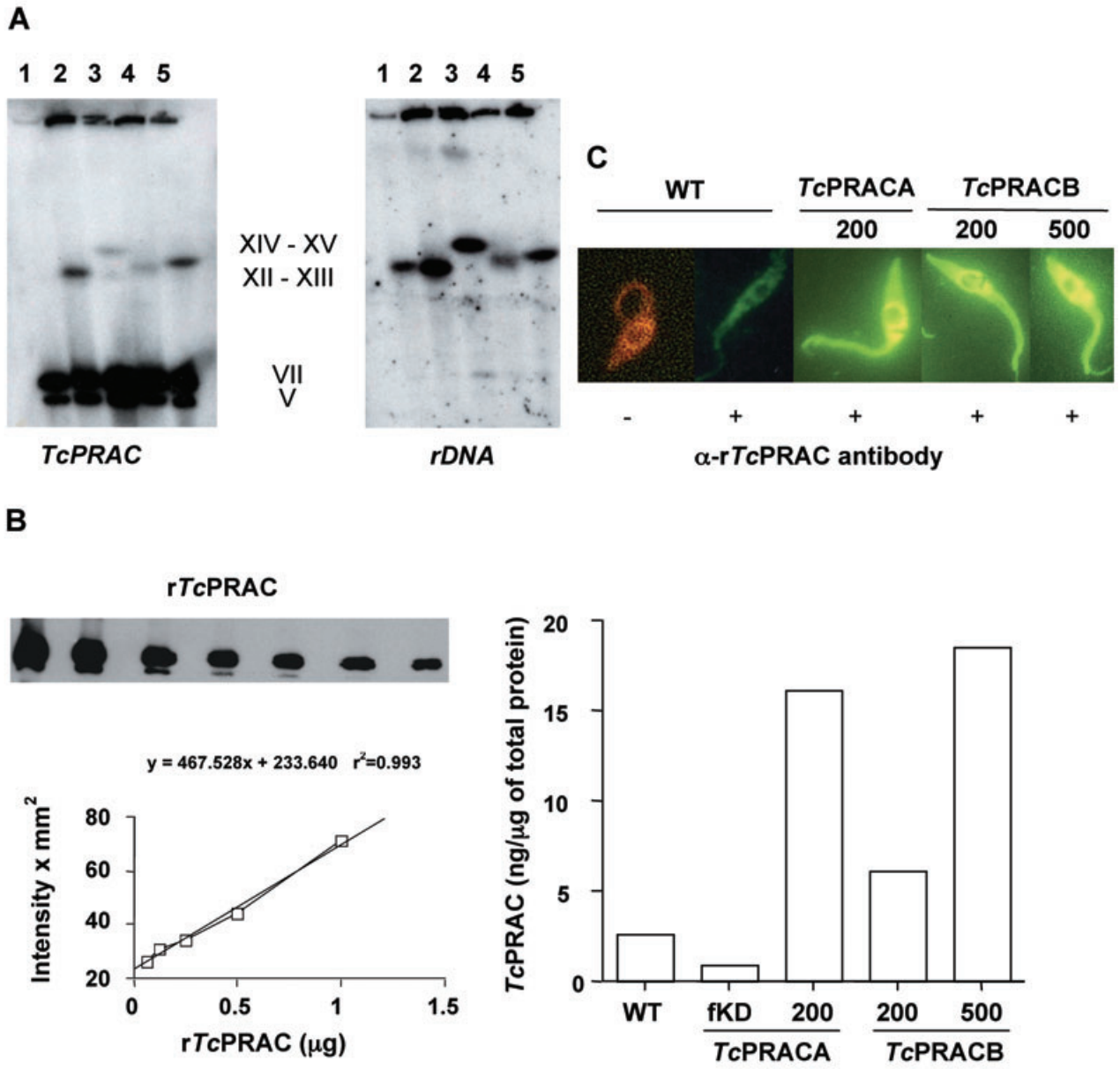

Fig. 2. Validation of the *TcPRAC* transgenesis strategy.
A. Chromosomal bands of wild type (lane 1), α*Tc*PRACA fKD (lane 2), *Tc*PRACA200 (lane 3), *Tc*PRACB200 (lane 4) and *Tc*PRACB500 (lane 5) epimastigotes after separation by pulsed-field gel electrophoresis and Southern blot hybridization with *Tc*PRAC probe (left) or rDNA probe (right). The chromosomal region numbers are indicated in roman numerals.
B. The evaluation of transgenesis in *Tc*PRACA and *Tc*PRACB in epimastigote non-infective forms of *T. cruzi* was performed by Western blot analysis using specific antibodies directed to *Tc*PRAC compared with known amounts of recombinant PR (left) and the QUANTITY ONE® program. Signal intensity of total protein lysates of epimastigotes transfected with pTREX-*TcPRACA* or pTREX-*TcPRACB* in the sense orientation is increased in respective clones obtained by limiting dilution. It should be noted that while parasites transfected with *TcPRACA* overexpression construct did not resist a selection pressure higher than 200 μg ml^{-1} of G418 (*Tc*PRACA200), it was possible to obtain two different lines of *TcPRACB* overexpressor parasites corresponding to medium (*Tc*PRACB200) or high (*Tc*PRACB500) degrees of PR production, respectively, derived from 200 μg ml^{-1} or 500 μg ml^{-1} of G418 pressure. Only epimastigotes transfected with antisense pTREX-*TcPRACA* construct were able to survive (fKD), contrasting with a complete lack of growth and viability obtained when epimastigotes were transfected with antisense pTREX-*TcPRACB* construct. Wild type (WT) epimastigotes were used as controls.
C. Immunolocalization of PR was performed by immunofluorescence using specific mouse polyclonal antibodies directed to *Tc*PRAC protein (α-r*Tc*PRAC) or with PBS (–). Reactions were revealed by Alexa Fluor™ 488 goat antibody against mouse IgG(H+L) F(ab′)$_2$. These experiments confirmed the presence of an intracellular isoform of PR whose localization is cytoplasmic and excluded from the nucleus or the kinetoplast in parasites overexpressing *TcPRACA* or *TcPRACB* (*Tc*PRACA200, *Tc*PRACB200 or *Tc*PRACB500) as compared with WT epimastigote.

sense orientation, were unable to grow under higher drug pressure indicating that overexpression, or molecular inactivation of *Tc*PRACA influenced parasite viability. Parasites transfected with both pTREX-*Tc*PRACA vectors and selected at 200 μg G418 ml^{-1} did give rise to viable epimastigotes and generated *Tc*PRACA200 overexpressor parasites and *Tc*PRACA functional knock-down parasites (α*Tc*PRACA fKD) respectively. Additionally, parasites transfected with pTREX-*Tc*PRACB vector in the antisense orientation were unable to survive in any drug condition indicating that viability of epimastigote forms is dependent on the expression of an intracellular isoform of PR. Nevertheless, transfected parasites with pTREX-*Tc*PRACB sense vector grew in both drug conditions (giving *Tc*PRACB200 and *Tc*PRACB500 parasite lines and clones). As expected, all constructs used were integrated into chromosomes containing the rDNA locus (Fig. 2A). It is noticeable that *Tc*PRACA200 present a different pattern of integration of the construct into the rDNA locus possibly due to the plasticity of *T. cruzi* genome. Nonetheless, as *TcPRACA* transgene presents a similar insertion environment to the other constructs, transgene expression should not in principle be affected if compared with that of the other transfectants. The levels of PR expression produced by the different transfectants were evaluated by Western blot analysis using total lysates obtained from equivalent numbers of parasites from the different cell lines and their respective clones. *Tc*PRAC production was evaluated in comparison with known amounts of recombinant *Tc*PRACA and *Tc*PRACB proteins using QUANTITY ONE® program (Fig. 2B, left). Thus, the original *Tc*PRACA200 overexpressor line and subsequent clones at low drug selection produced 10- to 20-fold increase in amounts of PR either relative to wild type (WT) parasites or relative to α*Tc*PRACA fKD. Furthermore, *Tc*PRACB200 and *Tc*PRACB500 parasite lines and their respective clones subjected to lower or higher antibiotic pressure showed 10- to 20- and 30-fold increased amounts of PR, respectively, as compared with WT parasites (Fig. 2B, right). To ascertain that differences observed in levels of expression were not due to the vector itself, we produced two cell lines transfected with pTREX-n vector alone and use two selection pressures of G418 (200 or 500 μg ml^{-1}). Western blot analysis of *Tc*PRAC production was similar to that of non-transfected WT parasites (data not shown). We then used specific antibodies directed to *Tc*PRAC to localize the protein by immunofluorescence in transfected parasites and respective clones. Although overexpressors of both PR genes present a brighter cytoplasmic staining than WT parasites, no staining is observed in the kinetoplast or in the nucleus indicating that increase in *Tc*PRAC expression does not interfere with protein localization inside the cell (Fig. 2C). To compare the relative amounts of *Tc*PRAC in low and high expressors, we performed a titration assay. Results presented in Table 1 show that α*Tc*PRACA fKD present a consistent absence of labelling at the same antibody concentration used for WT strain. In addition, using the same anti-recombinant *Tc*PRAC (α*rTc*PRAC) serum, we reproducibly observed higher titres on overexpressor clones. These data are in agreement with Western blot quantification (Fig. 2B). We next confirmed that levels of *Tc*PRAC protein expression are associated with specific mRNAs production. First, genomic DNA analysis of fragments obtained by PCR with specific PR gene primers and further submitted to GsuI restriction revealed that WT and transfected parasites present an unaltered digestion profile with bands corresponding to both genes (Fig. 3A). The direct identification of PR transcripts was then assessed by oligo(dT)-primed reverse transcription of total RNA obtained from WT and transfected parasites further amplified using SLp and RAB primers (Fig. 3B) and the specific sequence signatures of the two *TcPRAC* genes were confirmed by GsuI digestion (Fig. 3C). These results clearly indicate that specific production of *TcPRACA* or *TcPRACB* transcripts is responsible for *Tc*PRACA or *Tc*PRACB protein expression in transfected parasites.

Proline racemase involvement in T. cruzi *differentiation into infective forms*

To further determine the consequences of *TcPRAC* genes expression on the progression of *T. cruzi* development, mutant epimastigotes were differentiated *in vitro* into metacyclic trypomastigotes using a chemically defined axenic medium (Contreras *et al.*, 1985). Characteristic adhesion of differentiating parasite cells to culture flasks was observed for both WT and mutant parasites. Parasites were allowed to undergo complete metacyclogenesis and the highest yields of metacyclic trypomastigotes occurred progressively after 96–120 h of culture when parasite forms detached from the support. We then quantified metacyclic forms on light microscopic preparations stained with Giemsa that allowed a phenotypic characterization of parasite cells (Fig. 4A). Both metacyclic para-

Table 1. Titration of PR in the different life stages of *T. cruzi* by indirect immunofluorescence.

Parasite	Epimastigote	Metacyclic	Amastigote
Wild type	200	800	<400
α*Tc*PRACA fKD	<200	400	NA
*Tc*PRACA200	1600	25 600	>1600
*Tc*PRACB200	3200	12 800	>1600
*Tc*PRACB500	>3200	12 800	>1600

Immunofluorescence was performed using the polyclonal mouse serum against r*Tc*PRAC followed by staining with the Alexa Fluor™ 488 goat antibody against mouse IgG (H+L) $F(ab')_2$ fragment conjugate. Numbers indicate the last dilution of α-r*Tc*PRACA used that gives a positive signal as compared with control immunofluorescence without primary antibody.
NA, not available (see text).

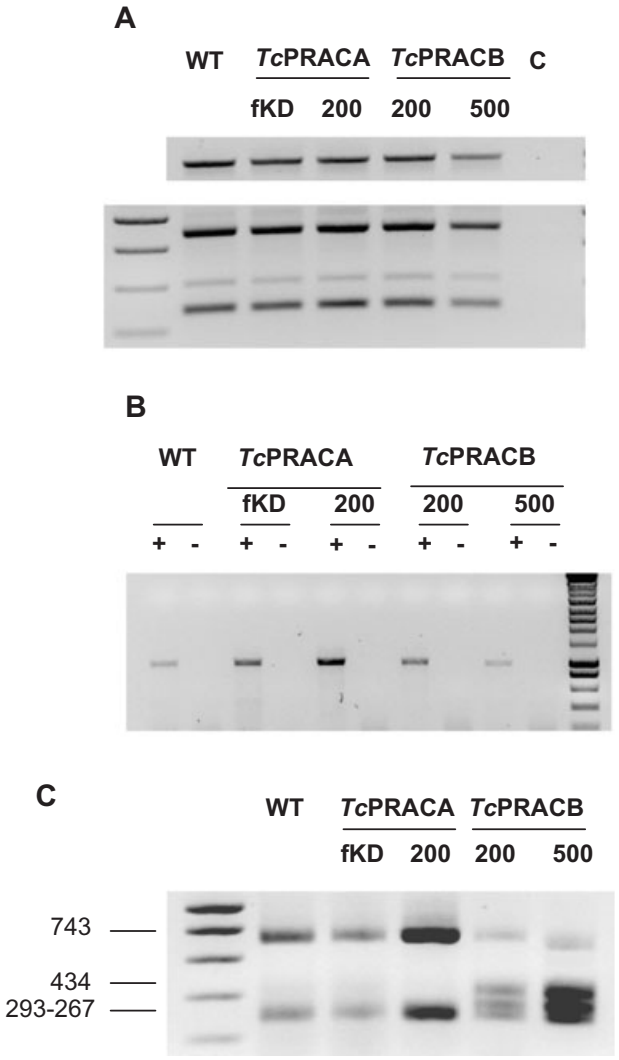

Fig. 3. RT-PCR analysis of polyA RNA from *TcPRACA* and *TcPRACB* transgenic parasites.
A. PCR amplification of genomic DNA with *TcPRAC* Hi/RAB-specific primers from wild-type (WT) and transgenic parasites for *TcPRACA* and *TcPRACB* genes (top), and GsuI enzyme restriction analysis of resultant fragments (bottom), fractionated on a 1.5% agarose gel containing ethidium bromide; C, internal negative control.
B. mRNA expression of *TcPRAC* in WT or transfected parasites shown by electrophoresis of gene fragments obtained by oligo(dT)-primed reverse transcription from total RNA extracted from equivalent parasite numbers, followed by PCR amplification using the sequences of the mini-exon (spliced leader, SLp) and RAB 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. RT-PCR amplified fragments were digested with GsuI and analysed on a 1.5% agarose gel containing ethidium bromide. Molecular sizes (kb) of the fragments are indicated.

sites, presenting an elongated morphology in which the kinetoplast migrated to the posterior end of the cells and intermediate forms, presenting an orthogonal position of the kinetoplast were counted. This set of experiments showed that PR plays a substantial role in parasite differentiation and that *Tc*PRACA and *Tc*PRACB proteins operate at different time points of metacyclogenesis (Fig. 4B and C). Thus, WT CL strain parasites display a typical metacyclogenesis where up to 30% of epimastigotes differentiated into non-dividing metacyclic forms when around 25% of the remaining parasites present an intermediate morphology. Parasites overexpressing *TcPRACB* gene, that generates only the intracellular PR isoform, display, however, similar levels of metacyclogenesis to WT but up to 50% of initial epimastigotes proceed to the first step of differentiation and present the kinetoplast apposed to the nucleus typical of the slender intermediate transition forms. This contrasts with the significantly improved metacyclogenesis accomplished by *Tc*PRACA overexpressors and with the seriously decreased (~50%) ability of α*Tc*PRACA fKD to differentiate.

Proline racemase implication in parasite invasion and development

Therefore, we designed a set of experiments to investigate the effect of PR overexpression on parasite infectivity and fate. Cell invasion assays were performed using confluent monolayer cultures of Vero cells subjected to 17 h of exposure to the different transgenic metacyclic parasites at 1:1 cell ratio. Scores of intracellular amastigotes 24 h post infection revealed that fully differentiated metacyclic forms of parasites overexpressing PR regardless of whether they carried *TcPRACA* (Fig. 5A) or *TcPRACB* (Fig. 5B) gene constructs are 5- to 10-fold more invasive to host cells than WT metacyclic CL strain parasites that presented in these experimental conditions the standard level of infectivity towards Vero cells in the order of 2%. Analysis of α*Tc*PRACA fKD invasion was, however, carried out using a 10-fold higher parasite/cell ratio to ensure that results would not be constrained by the low infectivity rate displayed by WT control parasites. As depicted in Fig. 5C the rate of infection of Vero cells cultured with α*Tc*PRACA fKD is nevertheless similar to that observed with WT metacyclic controls. We next used immunofluorescence assays either with specific antibodies directed to PR or with a hyperimmune serum recognizing all stages of *T. cruzi.* Ninety-six-hour cultures allowed us to evaluate the intracellular production of PR by amastigote forms before parasite release (Table 1) and consistently indicated an increased amount of amastigote forms of PR overexpressors in comparison with cellular infection with WT parasites (Fig. 5D). This clearly contrasted with the decreased number of amastigotes observed when using α*Tc*PRACA fKD. Based on these observations, additional experiments were conceived to evaluate parasite release from Vero cells. Considering the doubling time of intracellular amastigote forms our data showed that the number of

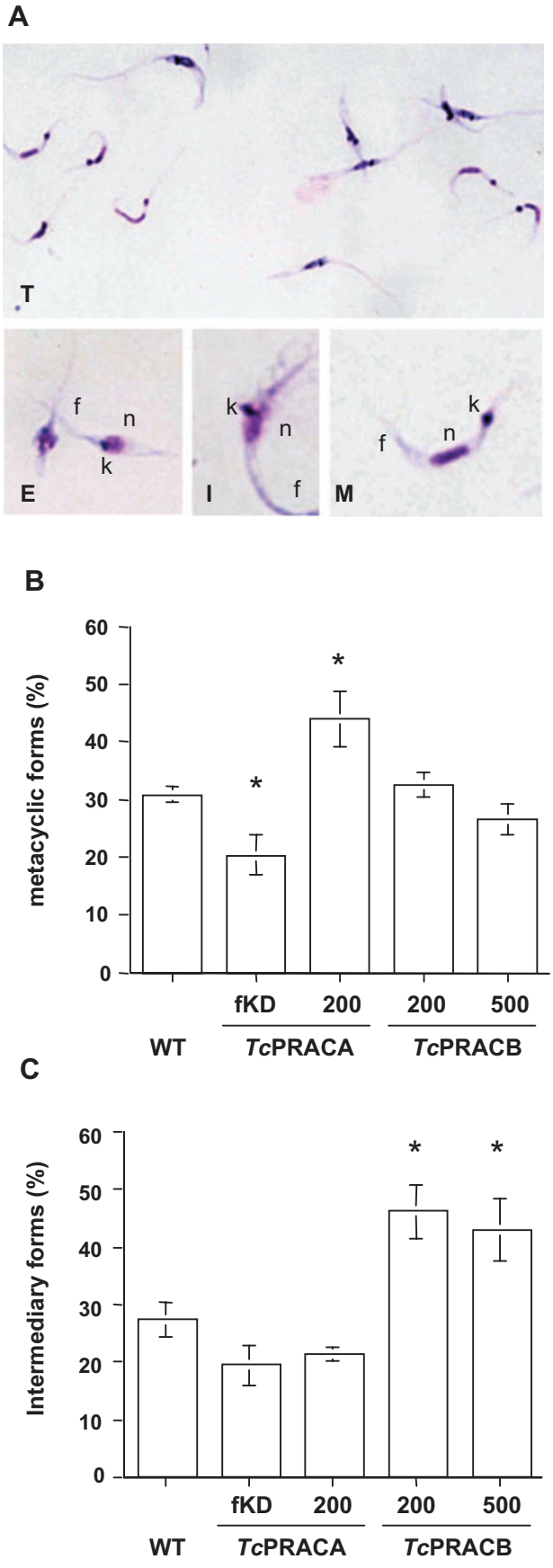

Fig. 4. Increased metacyclogenesis of PR overexpressors.
A. The efficiency of parasite metacyclogenesis was evaluated by the differential phenotype expressed by transgenic parasites, as described before (Contreras *et al*., 1985). Wild-type (WT) or transfected epimastigote forms obtained from log exponential phase cultures were washed and *in vitro* metacyclogenesis was induced in defined axenic TAU medium for 96–120 h. Parasites were then recovered from cultures and stained with Giemsa (T). Developmental stages of differentiation were scored by discriminating stout epimastigotes presenting a kinetoplast between the nucleus and the flagellum (E), intermediate slender forms where the kinetoplast is found apposite to the nucleus (I) and metacyclic long slender forms where the kinetoplast is subterminal (M).
B. The number of metacyclic forms obtained from cultures induced to undergo epimastigote differentiation of WT, *Tc*PRAC-overexpressing mutant lines (*Tc*PRACA200, *Tc*PRACB200 or *Tc*PRACB500) and the α*Tc*PRACA fKD mutant measured as a percentage of the total number of cells in the culture. Results show arithmetic means ± standard deviations of at least three experiments. $^*P < 0.05$ is shown whenever appropriate.
C. The number of intermediary forms obtained from cultures induced to undergo epimastigote differentiation of WT, *Tc*PRAC-overexpressing mutant lines (*Tc*PRACA200, *Tc*PRACB200 or *Tc*PRACB500) and the α*Tc*PRACA fKD mutant measured as a percentage of the total number of cells in the culture. Results show arithmetic means ± standard deviations of at least three experiments. $^*P < 0.05$ is shown whenever appropriate.

parasites released by Vero cells is directly proportional to the increase of cellular infection obtained with PR overexpressors contrasting to the number of parasites released by cultures infected with WT or with functional knock-down parasites and conclusively demonstrate that PR is a factor that contributes to *T. cruzi* infection outcome (Fig. 5E).

Discussion

We have previously identified a B-cell mitogen secreted by infective forms of *T. cruzi* that is also a PR (*Tc*PRAC) and showed that its expression as a cytoplasmic and/or secreted protein is life stage specific. Genomic analysis of *TcPRAC* indicated the presence of two paralogous genes per haploid genome (*TcPRACA* and *TcPRACB*). Our previous data have shown that both *TcPRACA* and *TcPRACB* encode functional PRs that display different kinetic parameters that can have implications in the efficiency of these enzymes in different parasite compartments or in the external milieu (Chamond *et al*., 2003). Initial analysis of the structure of *TcPRACA* and *TcPRACB* suggested that the two genes would, respectively, encode the secreted and cytoplasmic forms of PR. Closer examination revealed that *TcPRACA* possessed a second downstream *trans*-splice acceptor motif that if utilized would produce a cytoplasmic isoform of PR. Our data demonstrated that indeed *TcPRACA* is alternatively *trans*-spliced to produce mRNA species that encode cytoplasmic *Tc*PRACA. The secreted isoform of *Tc*PRACA is only expressed in metacyclic and trypomastigotes stages suggesting that the protein production is under develop-

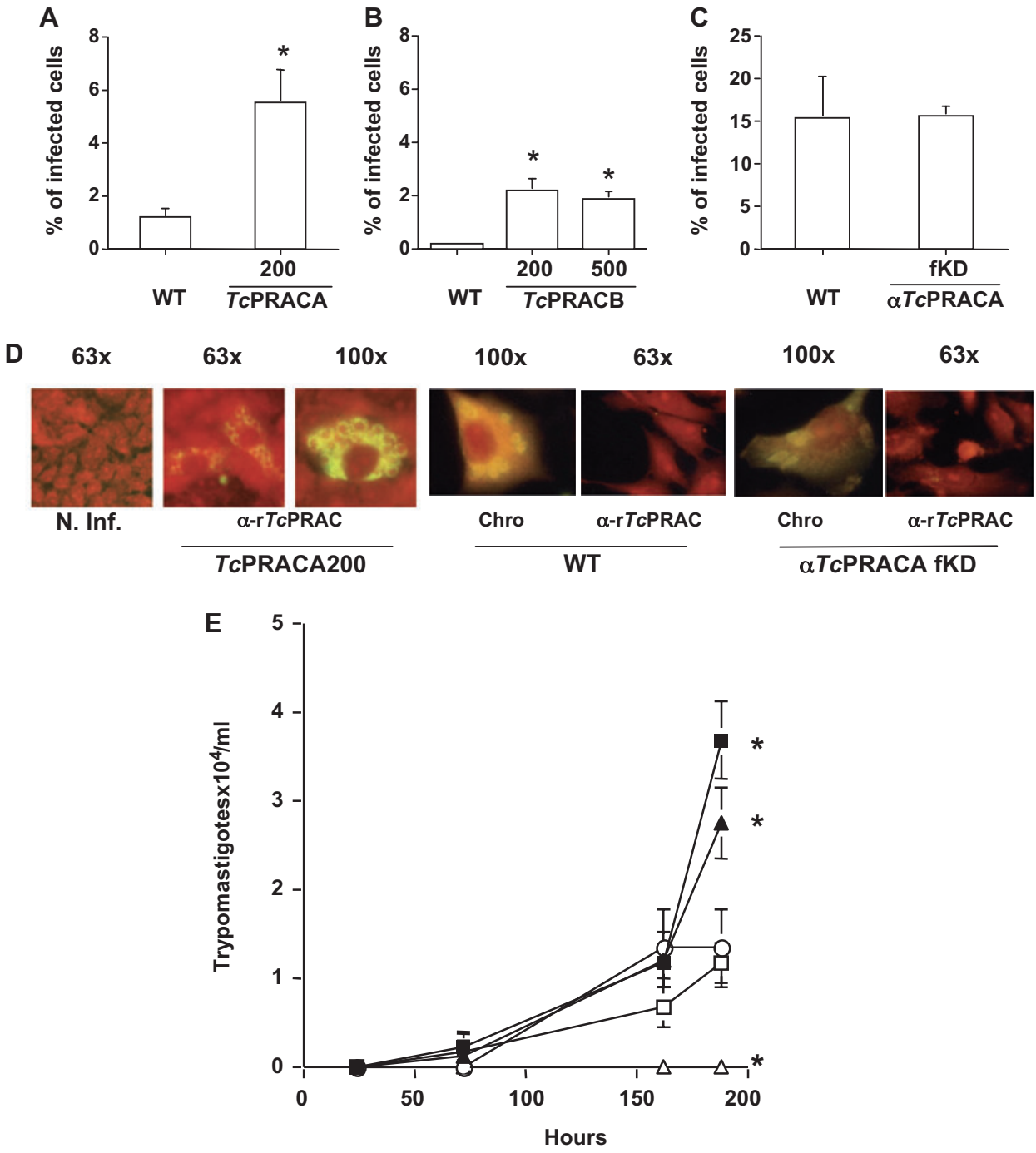

Fig. 5. *In vitro* infectivity of wild-type (WT) and transformed parasites.
A–C. The number of infected cells by WT, *Tc*PRAC-overexpressing mutant lines (*Tc*PRACA200, *Tc*PRACB200 or *Tc*PRACB500) and the α*Tc*PRACA fKD mutant measured as a percentage of the total number of cells in the culture. Vero cell cultures were incubated overnight with an equal number of purified metacyclic forms from WT or transgenic parasites at 1:1 parasite/cell ratio; extracellular parasites were then washed and intracellular amastigote forms scored at 24 h post infection. While a consistently higher infection of host cells was observed with *Tc*PRACA and *Tc*PRACB PR overexpressors; no differences in infection rate was observed between WT and α*Tc*PRACA fKD parasites at parasite/cell ratio reaching up to 10:1. **P* < 0.05 is shown whenever appropriate.
D. Immunofluorescence assays of 96 h Vero cell cultures infected with WT, *Tc*PRACA200 or α*Tc*PRACA fKD parasites. Parasites were stained with antibodies specific for *Tc*PRAC (α-r*Tc*PRAC) or chronic sera (Chro) from 8 months infected mice as indicated. WT or functional knock-down (α*Tc*PRACA fKD) metacyclic parasites were compared with non-infected (N. Inf.) cell cultures. Alexa Fluor™ 488 goat antibody against mouse IgG(H+L) $F(ab')_2$ was the secondary antibody. Microscopic magnifications are indicated (100× or 63×).
E. Release of trypomastigotes in the supernatants of cultures infected with *Tc*PRAC mutants: WT (○), *Tc*PRACA200 (▲), *Tc*PRACB200 (□), *Tc*PRACB500 (■) and α*Tc*PRACA fKD (△) metacyclic parasites. Results show arithmetic means ± standard deviations of at least three experiments. **P* < 0.05 is shown whenever appropriate.

mental control. Developmental regulation of *Tc*PRACA could be the consequence of a different stability of its transcripts during *T. cruzi* life cycle, transcripts encoding the secreted version of *Tc*PRACA being less stable in non-infective stages. Alternatively, *TcPRACA* transcripts encoding for the intracellular isoform of *Tc*PRACA could be constitutively produced whereas those encoding for the secreted isoform of *Tc*PRACA could only be efficiently *trans*-spliced in infective stages. This hypothesis predicts that alternative *trans*-splicing is developmentally regulated. Alternative *trans*-splicing has only been reported once in *T. cruzi*. The *LYT1* gene is also predicted to produce secreted and non-secreted protein isoforms in a developmentally regulated fashion (Manning-Cela *et al*., 2002). Thus, it is possible for *trans*-splicing signals to be embedded within protein coding regions of a gene and a re-examination of the potential of the *T. cruzi* genome to encode splice variants is warranted. In addition, the organization of *TcPRACA* provides a trypanosomatid alternative to the general eukaryotic mechanism of differential promoter selection and *cis*-splicing for the production of an intracellular or a secreted version of a protein. Therefore, *TcPRACA*-like gene organization would permit stringent conservation and utilization of a constant catalytic domain in different biological compartments.

It has been demonstrated that the secreted form of *Tc*PRAC is both an enzyme and a mitogen (Reina-San-Martin *et al*., 2000) and that the intracellular isoform has distinct biochemical and enzymatic properties (Chamond *et al*., 2003). Through the generation of different mutant parasites that were either α*Tc*PRAC functional knockdown (fKD) or *Tc*PRAC-overexpressing mutants in non-infectious epimastigote forms, we revealed additional roles of *Tc*PRAC. Therefore, expression of full-length *TcPRACA* and *TcPRACB* was undertaken using a validated expression vector, pTREX-n, which was also used to produce mutant parasites expressing full-length antisense RNA (α*TcPRACA* and α*TcPRACB* respectively) to both genes, an approach that has been successful previously (Allaoui *et al*., 1999). However, α*Tc*PRACB parasites were not viable, and α*Tc*PRACA fKD parasites survived only under low selection pressure. This suggests that the antisense strategy can discriminate between the two transcripts and that *TcPRACB* is essential in epimastigotes. One explanation might be that expression of α*TcPRACA* at low G418 concentrations would only efficiently interfere with *TcPRACA* transcripts leaving functional *TcPRACB* transcripts. However, the levels of α*TcPRACA* expression selected by the highest G418 pressure are lethal because both *TcPRACA* and *TcPRACB* transcripts would be targeted. This conclusion is supported by the viability of α*Tc*PRACA parasites at low G418 concentration despite a demonstrable reduction in the total amount of epimastigote *Tc*PRAC. Furthermore, the viability of α*Tc*PRACA parasites argues for distinct functions of *Tc*PRAC isoforms which is consistent with their different functional characteristics (Chamond *et al*., 2003).

Overexpression of both *Tc*PRAC isoforms was possible and dose dependent for *TcPRACB* but again only possible in parasites grown at the lowest drug concentration for *TcPRACA*. Overexpression of both isoforms influenced normal epimastigote differentiation but in different manners. *Tc*PRACA200 parasites undergo increased metacyclogenesis while a high proportion of *Tc*PRACB200/500 parasites delay differentiation in intermediate stages again implying distinct roles for *Tc*PRAC isoforms in epimastigote differentiation. Signals for differentiation in the insect gut are a decrease in gut glucose levels paralleled by an increase in parasite L-proline consumption. These changes accompany the initiation of parasite attachment and flagellum enlargement in the insect rectum where the slightly acidic pH content changes to alkaline pH in the urine after feeding causing variations of environmental osmolality that favour parasite differentiation (Kollien and Schaub, 2000; Kollien *et al*., 2001). The role of L-proline was recently reinforced by a study demonstrating that intracellular *T. cruzi* differentiation into trypomastigotes is *bona fide* dependent on proline concentration (Tonelli *et al*., 2004). We have previously shown that recombinant *Tc*PRACB reflects the native intracellular protein and displays higher enzymatic activity and across a broader pH range (4.5–8.5) than recombinant *Tc*PRACA (5.5–7.0) (Chamond *et al*., 2003). This suggests that *Tc*PRACB would contribute the most significant PR enzymatic activity during epimastigote differentiation and that the contribution of the *Tc*PRACA isoform would be transient and mostly involved in the later steps of epimastigote differentiation, supporting the enlargement of flagellum and ultimate kinetoplast migration and stabilization in the posterior end of the cell. The observation of a drastic increase in parasites in the intermediate stage of differentiation while *Tc*PRACB is overexpressed corroborates the existence of such a stage during epimastigote to metacyclic differentiation and parallel similar observations made during the amastigote to trypomastigote differentiation (Almeida-de-Faria *et al*., 1999; Tyler and Engman, 2001). Furthermore, recent work performed on amastigote to trypomastigote differentiation revealed that L-proline concentration was crucial for *T. cruzi* differentiation in the mammalian host (Tonelli *et al*., 2004) thus favouring our hypothesis about the importance of L-proline as an environmental stimulus and more importantly about PRs as key enzymes in environmental sensing.

In addition, as more than 18 different intermediate stages of *T. cruzi* could be classified in the vector (Chagas, 1909; Schaub, 1989; Perlowagora-Szumlewicz and Moreira, 1994), and feeding or starvation may induce drastic changes and effects on insect gut conditions and

consequently on metacyclogenesis, the existence of two significantly different isoforms of PR enzymes would afford the parasite a flexible ability to differentiate and acquire infectivity.

Although it is only possible to speculate on functions of this intracellular version of PR, recent data on the existence of proline transport systems in *T. cruzi* indicates that PR may well be central to parasite bioenergetics (Silber *et al*., 2002) as proline is known to be an important energy source in trypanosomatids (Sylvester and Krassner, 1976) and several other organisms (Evans and Brown, 1972; Krassner and Flory, 1972). The essential nature of an intracellular isoform of PR in the cytoplasm of vector non-infective epimastigote stage is interesting as differentiation into infective metacyclic parasites occurs in the insect gut where proline is the most abundant free amino acid (de Isola *et al*., 1981). Thus, in the presence of active L-proline uptake PR would make D-amino acids available in the parasite cell. While ribosome-dependent pathways of D-amino acid inclusion in protein generation have not yet been demonstrated, it is conceivable that PR may contribute to post-translational addition of D-proline enantiomers to protein chains displayed by the parasite. As previously described in other systems (Mignogna *et al*., 1998), this hypothesis would certainly benefit infective parasite forms by guaranteeing enhanced resistance to host proteolytic enzymes whose target need to have only a single D-amino acid residue in order to become resistant to proteolysis (Janeway and Humphrey, 1969). In contrast to other trypanosomatids, *T. cruzi* penetration into non-phagocytic mammalian cells occurs by the recruitment and fusion to host lysosomes at the parasite attachment site followed by the formation of a parasitophorous vacuole that bursts shortly after invasion allowing the parasites to differentiate into amastigotes in the cytosol of host cells (Tardieux *et al*., 1992; Rodriguez *et al*., 1996; Reddy *et al*., 2001). Although not yet proven, it is clear that the presence of D-proline in proteins exposed by *T. cruzi* would favour parasite persistence and multiplication inside the host cytoplasm through enhanced lack of susceptibility to host proteases delivered by the lysosomes.

The presumed role of *Tc*PRACA isoform in parasite virulence was resolved by the use of the *Tc*PRACA200 parasites. *Tc*PRACA200 parasites that underwent metacyclogenesis both were more invasive to host cells and released higher numbers of trypomastigotes into the milieu once the amastigotes had replicated and differentiated in the host cell cytosol. Thus, *Tc*PRACA is both a mitogen and a virulence factor. Although the potentiation of virulence is mechanistically unclear it is tempting to hypothesize that PR may be involved in the modification of host cell membrane molecules facilitating adhesion and subsequent parasite penetration and development. *T. cruzi* host cell invasion is dependent on heparan sulphate receptors, heparin and collagen, that are the main components of the host extracellular matrix (Ortega-Barria and Pereira, 1991; Herrera *et al*., 1994). Epimerases, mitogens and growth factors are all involved in the attachment of heparan sulphate chains to core molecules of cardiovascular cells (Rosenberg *et al*., 1997) that are preferential targets for *T. cruzi*. Therefore, the involvement of a secreted mitogenic PR in modification of the target cell may well be envisaged. This is supported by our preliminary data: the addition of purified PR to supernatants of host cell cultures improves WT parasite infectivity and fate in a dose-dependent manner (not shown). Previous work has shown that r*Tc*PRACA is mitogenic *in vivo* as are metacyclic parasites (Reina-San-Martin *et al*., 2000). Therefore, we would predict that the threshold of parasite mitogenicity *in vivo* will be induced by a relatively smaller number of transgenic *Tc*PRACA200 metacyclic parasites and a relatively larger number of α*Tc*PRACA fKD parasites. Alternatively, but not exclusively, our data cannot exclude the hypothesis that *Tc*PRAC proteins would be involved in the mechanisms affecting parasite multiplication and amastigote differentiation. Although this possibility is attractive, its assessment is technically complex as the number of parasite per infected cell varies and the attribution of multiplication rates is dependent on synchronous and uniform levels of host cells infectivity. However, our data could not reveal any increase in parasite release other than the amplification effect due to enhanced cell invasion, favouring the hypothesis that cell penetration is the limiting step affected by genetic manipulation of *TcPRAC* genes.

Finally, we recently obtained the crystallographic structure of *T. cruzi* PR that combined with site-direct mutagenesis allowed us to identify amino acid residues that are critical for enzyme activity and thus propose a new mechanism for PR activity that is distinct from the current racemization reaction proposed by theoretical analysis (Rudnick and Abeles, 1975; Fisher *et al*., 1986) of the bacterium *Clostridium sticklandii* PR (M. Goytia *et al*., in preparation). Furthermore, the definition of a putative peptide signature for PRs permitted us to identify several candidate genes in the genome of distinct organisms of medical and agricultural interest, yet absent in mammalian host (Chamond *et al*., 2003), consequently suggesting that the design of inhibitors to this eukaryotic parasite enzyme may have a broad therapeutic potential in addition to the previously demonstrated, direct therapeutic value of *Tc*PRAC as a vaccine (Reina-San-Martin *et al*., 2000; Chamond *et al*., 2002).

Experimental procedures

Parasites cultures and metacyclogenesis

Trypanosoma cruzi, clone CL Brener, was used throughout

this study (http://www.pasteur.fr/recherche/unites/tcruzi/minoprio/TcruziDB/clbrener.html). Non-infective epimastigotes were maintained at 28°C by weekly passages in liver infusion tryptose (LIT) medium 10% fetal calf serum (FCS) alone or supplemented whenever indicated. Metacyclogenesis was performed by incubating log phase epimastigote forms of WT or transfected parasites in Triatome Artificial Urine medium (TAU medium) as described previously (Contreras *et al*., 1985). Optimum rate of epimastigote differentiation for the CL strain was considered as conversion of 30% to metacyclic forms by 96–120 h culture. Purification of metacyclic forms was performed, as described (de Sousa, 1983). Differentiation of epimastigotes into metacyclic forms was ascertained by light microscopy of parasite preparations stained with Giemsa (10% w/v in water) and evaluation of the morphology and the kinetoplast position. Culture trypomastigote forms were obtained from culture supernatants of infected Vero cells maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Life Technologies, Paisley, Scotland) supplemented with 10% FCS. Intracellular amastigote forms were analysed after 96 h culture in Vero cells previously infected with metacyclic parasites at 1:1 cell ratio. Bloodstream trypomastigote forms were obtained from C3H/HeJ mice used for serial passage of the parasite *in vivo*.

Parasite DNA and RNA preparations and GsuI restriction assays

TcPRACA or *TcPRACB* individual phage DNA (Reina-San-Martin *et al*., 2000) and genomic DNA from WT or transgenic parasites were obtained by PCR. *TcPRAC*-specific pair of common primers for both *TcPRACA* and *TcPRACB* genes were (Hi-45): 5′-CTCTCCCATGGGGCAGGAAAAAGCTT CTG-3′ and (RAB): 5′-TCGTACACAAAAGTCTCTCC-3′. PCR conditions for amplification were: 4 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 58°C, 2.5 min at 68°C, followed by 10 min at 68°C. PCR products were purified with the Qiagen PCR extraction kit (Qiagen, Courtaboeuf, France). RNA was extracted from epimastigote, purified metacyclic, bloodstream and culture trypomastigote forms using TRIzol reagent (Invitrogen, Life Technologies) following the manufacturer's instructions. Reverse transcription was performed with SuperScript II (Invitrogen, Life Technologies) using the antisense Oligo(dT) primer (dTun): 5′-pd$(T)_{20}$(G/A/C)(A/C/G/T)-3′ and corresponding cDNAs amplified by PCR using Pfu polymerase (Stratagene, Saint Quentin en Yvelines, France) and the following primers: (SLp, for spliced leader primer): 5′-AACGCTATTATTGATACAGTTTC-3′ and (RAB, for common reverse A/B sequence primer). PCR conditions were: 4 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 58°C, 2.5 min at 68°C, followed by 10 min at 68°C. (SLp/RAB) obtained fragments were purified with the Qiagen PCR extraction kit (Qiagen) and used for cloning into pCR-Blunt II-TOPO vector using the Zero blunt TOPO PCR cloning kit (Invitrogen, Life Technologies) following the manufacturer's instructions. Recombinant clones were screened and sent for sequencing (at Genome Express, Meylan, France). Purified genomic DNA, phage DNAs or SLp/RAB fragments obtained by RT-PCR were digested with GsuI during 2 h at 30°C. Profiles were analysed on 1–1.5% agarose gels containing ethidium bromide.

Generation of transgenic parasites for TcPRACA *or* TcPRACB *gene*

Full length of *TcPRACA* and *TcPRACB* coding gene sequences (GenBank Accession No. AF195522 and AY140947; Reina-San-Martin *et al*., 2000; Chamond *et al*., 2003) was generated by PCR using the respective phage DNAs and the specific set of primers: (Nc) 5′-GGGTCC ATGGGTAAAAGTGTCTGTCC-3′ and (Bg-45) 5′-CTGAG CTCGACCAGAT(CA)TACTGC-3′, for *TcPRACA* (codons +2 to +429) and (Hi-45) 5′-CTCTCCCATGGGGCAGGAAAAGCT TCTG-3′ and (Bg-45) primer for *TcPRACB* (codons +38 to +360). Reactions consisted of 25 cycles at 94°C (45 s), 53°C (45 s) and 72°C (2.5 min). PCR products were purified as prescribed by Qiagen PCR extraction kit (Qiagen). The purified blunt-ended *TcPRACA* Nc-Bg insert of 1294 bp was cloned into the HincII site of the pTREX-n vector (Vazquez and Levin, 1999) to generate pTREX-*TcPRACA* construct. *TcPRACB* Hi-Bg insert of 1195 bp was digested with HindIII (1176 bp) and ligated to HindIII/HincII sites of pTREX-n vector to generate pTREX-*TcPRACB* construct. DH5α bacteria were transformed with pTREX-*TcPRACA* (predicted 7556 bp), pTREX-*TcPRACB* (predicted 7438 bp) or pTREX-n constructs and respective DNA minipreps of the corresponding plasmids were purified using Qiagen Miniprep Kit (Qiagen). The restriction pattern obtained after HindIII or BamHI digestions of the purified DNA inserts allowed the selection of pTREX-*TcPRACA* constructs based on the sense or antisense orientation of their fragment revealed by size analysis of the obtained fragments in 0.8% agarose gels containing ethidium bromide. Antisense pTREX-*TcPRACB* vector was prepared by digestion of sense pTREX-*TcPRACB* construct with HindIII and XhoI to rescue *TcPRACB* gene fragment that was blunt-ended with Klenow polymerase and recloned in pTREX-n previously digested by HincII. Antisense orientation of the fragment was confirmed by BamHI digestion. A total of 5×10^8 *T. cruzi* log phase epimastigote forms were collected by centrifugation, washed and resuspended in Zimmerman post-fusion medium (Bellofatto and Cross, 1989), according to Hariharan *et al*. (1993). Parasite suspension was electroporated twice with 1.5 KV, 25 μF (Gene Pulser II, Bio-Rad) with 10 μg of DNA from each of the previously purified pTREX-n, pTREX-*TcPRACA* and pTREX-*TcPRACB* sense and antisense vectors in 0.4 cm gap electroporation cuvettes. Electroporated cells were diluted in LIT medium and incubated at 28°C overnight. Parasites were then selected for growth in selective medium containing 200 μg ml^{-1} or 500 μg ml^{-1} geneticine (G418). Cultures of parasites transfected with antisense constructs were firstly supplemented with 5 mM L- and D-proline. Non-transfectants were demonstrated after 1 week culture in selective medium. Transgenic epimastigotes were then cloned by limiting dilution and clones were maintained by weekly passage in LIT medium under drug pressure and integration was efficiently assessed by parasite chromoblots using, respectively, Hi/Bg *Tc*PRAC fragment, *Neo*, *gapdh* intergenic region and *rDNA* gene spacer fragment-specific probes (Martinez-Calvillo *et al*., 1997) (see below).

Western blots

Recombinant *Tc*PRACA, r*Tc*PRACB (Reina-San-Martin

et al., 2000; Chamond *et al.*, 2003), total extracts obtained from equivalent numbers of WT or transgenic parasites were separated by gradient SDS-PAGE (4–12% or 8–12%) and proteins were electrophoretically transferred onto nitrocellulose membranes. When indicated, total protein concentration of samples were adjusted to 300 $\mu g\ ml^{-1}$. Membranes were saturated in Tris-buffered saline and milk, washed, incubated with polyclonal serum against r*Tc*PRAC, washed and developed with peroxidase-labelled secondary antibody using chemiluminescence (ECL kit, Amersham, Orsay, France). Blots were scanned and analysed with QUANTITY ONE® program.

Immunofluorescence assays

Indirect immunofluorescence was used in cellular localization, titration and infectivity assays. Epimastigote, metacyclic and intracellular amastigotes of WT or transgenic parasites were incubated with polyclonal mouse serum directed to r*Tc*PRAC followed by 4 $\mu g\ ml^{-1}$ Alexa Fluor™ 488 goat antibody against mouse IgG(H+L) $F(ab')_2$ fragment conjugate (Molecular Probes Interchim, Montluçon, France). Control stainings were performed using Alexa Fluor™ 488 goat antibody against mouse IgG(H+l) $F(ab')_2$ fragment conjugate alone or after incubation of the parasites with chronic serum obtained from mice infected with the parasite for 8 months.

Chromoblots and Southern blots

Agarose (0.7%) blocks containing 1×10^7 cultured epimastigotes forms of *T. cruzi* CL Brener or transfected parasites were lysed with 0.5 M EDTA/10 mM Tris/1% sarcosyl pH 8.0, digested by proteinase K and washed in 10 mM Tris/1 mM EDTA, pH 8.0. Pulsed field gel electrophoresis (PFGE) was carried out at 18°C using the Gene Navigator apparatus (Pharmacia, Upsala, Sweden) in 0.5× TBE. Electrophoresis was performed, as described in (Cano *et al.*, 1995). Gels were then stained with ethidium bromide, exposed to UV light (265 nm) for 5 min and further blotted under alkaline conditions to positive TM membrane (Q BIO gene). Hi/Bg *TcPRAC* probe was obtained by PCR amplification of *TcPRAC* gene with Hi-45 and Bg-45 common primers (as described in Reina-San-Martin *et al.*, 2000) and *Neo* probe with neo-sense (5′-CTTGGGTGGAGAGGCTATTC-3′) and neo-antisense (5′-AGGTGAGATGACAGGAGATC-3′) primers. *gapdh* intergenic region and *rDNA* gene spacer fragment probes were obtained by appropriate digestions of pTREX-n vector. Probes were purified and labelled with [α-^{32}P]-dCTP using Megaprime DNA labelling system (Amersham Life Science). The blots were hybridized with resulting probes 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).

Parasite entry and infection assays

Parasite entry was accessed in cultures of confluent monolayers of 2×10^5 Vero cells ml^{-1} DMEM medium containing 10% FCS seeded on Laboratory-tek chamber slides (Nalge Nunc International, Naperville, IL) and infected at 1:1 ratio with 2×10^5 metacyclic (or 10:1 ratio, whenever indicated) with WT or transgenic metacyclic parasites at 37°C, under 5% CO_2. Extracellular parasites were washed from the cultures 17 h later with pre-warmed medium. At 24 h chambers were washed with PBS and preparations were fixed with Zincker/acetic acid solution. Slides were further stained by Lugol and fast Giemsa (Réactifs RAL Bordeaux Technopolis, Martillac, France). Two thousand cells per chamber were counted and intracellular amastigote forms were determined by light microscopy. Additional cultures of Vero cells were seeded at $5 \times 10^4\ ml^{-1}$ and incubated with metacyclic trypomastigotes (1:1 ratio); extracellular parasites were washed out from the cultures 17 h later with pre-warmed medium and WT or transgenic metacyclic parasites released in the supernatants of ongoing cultures were counted thereafter during 8 days, as indicated.

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