**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 *TcPRAC* 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 *TcPRAC* 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...
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 (TcPRACA) 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 TcPRACA 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 TcPRACA and TcPRACB 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 poly-pyrimidine-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 preceed 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 Gsu 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). Gsu 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 TcPRACA 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 TcPRAC 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-interactive 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 anti-sense orientations to obtain, respectively, transfectants overexpressing TcPRACA and TcPRACB, or potentially functional knock-down parasites for natural TcPRAC. Transfected parasites were selected using two drug concentrations (200 and 500 µg 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-TcPRACA vectors, either in the sense or anti-sense orientations, were unable to survive in culture. However, we managed to obtain parasites transfected with TcPRACB vectors, which were able to grow in culture. In these parasites, RT-PCR analysis revealed the presence of TcPRACB as a processed transcript, indicating that both TcPRACB and TcPRACA are transcribed in all the parasite life stages analysed, and that the transcription of TcPRACB is more efficient than that of TcPRACA. This suggests that TcPRACB is a more suitable target for overexpression in T. cruzi parasites.

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 poly pyrimidine-rich-signal domains (CT₅₉). 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 (−27) 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 A/AG of TcPRACB sequence. 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.

B. The digestion of (Hi/RAB) fragments obtained by PCR of DNA from original individual phagings 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 TcPRACA and TcPRACB 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.

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Fig. 2. Validation of the TcPRAC transgenesis strategy.
A. Chromosomal bands of wild type (lane 1), αTcPRACA fKD (lane 2), TcPRACA200 (lane 3), TcPRACB200 (lane 4) and TcPRACB500 (lane 5) epimastigotes after separation by pulsed-field gel electrophoresis and Southern blot hybridization with TcPRAC probe (left) or rDNA probe (right). The chromosomal region numbers are indicated in roman numerals.
B. The evaluation of transgenesis in TcPRACA and TcPRACB in epimastigote non-infective forms of T. cruzi was performed by Western blot analysis using specific antibodies directed to TcPRAC 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 (TcPRACA200), it was possible to obtain two different lines of TcPRACB overexpressor parasites corresponding to medium (TcPRACB200) or high (TcPRACB500) 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 TcPRAC protein (α-rTcPRAC) 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 (TcPRACA200, TcPRACB200 or TcPRACB500) as compared with WT epimastigote.
sense orientation, were unable to grow under higher drug pressure indicating that overexpression, or molecular inactivation of TcPRACA influenced parasite viability. Parasites transfected with both pTREX-TcPRACA vectors and selected at 200 μg G418 ml⁻¹ did give rise to viable epimastigotes and generated TcPRACA200 overexpressor parasites and TcPRACA functional knock-down parasites (αTcPRACA fKD) respectively. Additionally, parasites transfected with pTREX-TcPRACB 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-TcPRACB sense vector grew in both drug conditions (giving TcPRACB200 and TcPRACB500 parasite lines and clones). As expected, all constructs used were integrated into chromosomes containing the rDNA locus (Fig. 2A). It is noticeable that TcPRACA200 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. TcPRAC production was evaluated in comparison with known amounts of recombinant TcPRACA and TcPRACB proteins using QUANTITY ONE® program (Fig. 2B, left). Thus, the original TcPRACA200 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 αTcPRACA fKD. Furthermore, TcPRACB200 and TcPRACB500 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 TcPRAC production was similar to that of non-transfected WT parasites (data not shown). We then used specific antibodies directed to TcPRAC 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 TcPRAC expression does not interfere with protein localization inside the cell (Fig. 2C). To compare the relative amounts of TcPRAC in low and high expressors, we performed a titration assay.

Results presented in Table 1 show that αTcPRACA fKD present a consistent absence of labelling at the same antibody concentration used for WT strain. In addition, using the same anti-recombinant TcPRAC (αTcPRAC) 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 TcPRAC 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 TcPRACA or TcPRACB 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.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Epimastigote</th>
<th>Metacyclic</th>
<th>Amastigote</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>200</td>
<td>800</td>
<td>&lt;400</td>
</tr>
<tr>
<td>αTcPRACA fKD</td>
<td>&lt;200</td>
<td>400</td>
<td>NA</td>
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<tr>
<td>TcPRACA200</td>
<td>1600</td>
<td>25 600</td>
<td>&gt;1600</td>
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<tr>
<td>TcPRACB200</td>
<td>3200</td>
<td>12 800</td>
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<td>TcPRACB500</td>
<td>&gt;3200</td>
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Immunofluorescence was performed using the polyclonal mouse serum against rTcPRAC 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-rTcPRACA used that gives a positive signal as compared with control immunofluorescence without primary antibody. NA, not available (see text).
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 TcPRACA and TcPRACB 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 TcPRACA overexpressors and with the seriously decreased (~50%) ability of αTcPRACA 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 αTcPRACA 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 αTcPRACA 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 αTcPRACA 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

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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 (*TcPRAC*) 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 *TcPRACA*. The secreted isoform of *TcPRACA* 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, TcPRAC-overexpressing mutant lines (TcPRACA200, TcPRACB200 or TcPRACB500) and the αTcPRACA 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 TcPRAC and TcPRACB PR overexpressors; no differences in infection rate was observed between WT and αTcPRACA 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, TcPRACA200 or αTcPRACA fKD parasites. Parasites were stained with antibodies specific for TcPRAC (α-rTcPRAC) or chronic sera (Chro) from 8 months infected mice as indicated. WT or functional knock-down (αTcPRACA 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 TcPRAC mutants: WT (○), TcPRACA200 (▲), TcPRACB200 (□), TcPRACB500 (■) and αTcPRACA 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 TcPRACA could be the consequence of a different stability of its transcripts during T. cruzi life cycle, transcripts encoding the secreted version of TcPRACA being less stable in non-infective stages. Alternatively, TcPRACA transcripts encoding for the intracellular isoform of TcPRACA could be constitutively produced whereas those encoding for the secreted isoform of TcPRACA 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 TcPRAC 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 αTcPRAC functional knock-down (fKD) or TcPRAC-overexpressing mutants in non-infectious epimastigote forms, we revealed additional roles of TcPRAC. 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 (αTcPRACA and αTcPRACB respectively) to both genes, an approach that has been successful previously (Allaoui et al., 1999). However, αTcPRACB parasites were not viable, and αTcPRACA 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 αTcPRACA at low G418 concentrations would only efficiently interfere with TcPRACA transcripts leaving functional TcPRACB transcripts. However, the levels of αTcPRACA 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 αTcPRACA parasites at low G418 concentration despite a demonstrable reduction in the total amount of epimastigote TcPRAC. Furthermore, the viability of αTcPRACA parasites argues for distinct functions of TcPRAC isoforms which is consistent with their different functional characteristics (Chamond et al., 2003).

Overexpression of both TcPRAC 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. TcPRACA200 parasites undergo increased metacyclogenesis while a high proportion of TcPRACB200/500 parasites delay differentiation in intermediate stages again implying distinct roles for TcPRAC 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 TcPRACB reflects the native intracellular protein and displays higher enzymatic activity and across a broader pH range (4.5–8.5) than recombinant TcPRACA (5.5–7.0) (Chamond et al., 2003). This suggests that TcPRACB would contribute the most significant PR enzymatic activity during epimastigote differentiation and that the contribution of the TcPRACA 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 TcPRACB 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 proteinolysis (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 *TcPRACA* isoform in parasite virulence was resolved by the use of the *TcPRACA*200 parasites. *TcPRACA*200 parasites that underwent metacyclogenesis both were more invasive to host cells and released higher numbers of trypmastigotes into the milieu once the amastigotes had replicated and differentiated in the host cell cytosol. Thus, *TcPRACA* 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*TcPRACA* 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 *TcPRACA*200 metacyclic parasites and a relatively larger number of α*TcPRACA*1KD parasites. Alternatively, but not exclusively, our data cannot exclude the hypothesis that *TcPRAC* 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 racemase signature for PRs permitted us to identify several candidate signature for PRs permitted us to identify several candidate signature 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 *TcPRAC* as a vaccine (Reina-San-Martin et al., 2000; Chamond et al., 2002).

**Experimental procedures**

**Parasites cultures and metacyclogenesis**

*Tryptosoma cruzi*, clone CL Brener, was used throughout...
by 10 min at 68°C, cycles of 45 s at 94°C. PCR conditions for amplification were: 4 min at 94°C, followed by 35 cycles of 45 s at 94°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).

Bloodstream trypanosome DNA was extracted 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 trypanosome DNA was 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.

**Parasite DNA and RNA preparations and Gsul 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'-CTCTCCATGGGGCAGGAAAAAGCTTCTG-3' and (RAB): 5'-TCGTACACAAAGGTCTCTTCC-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 trypanosome forms using TRIzol reagent (Invitrogen, Life Technologies) following the manufacturer's instructions. Transcription was performed with SuperScript II (Invitrogen, Life Technologies) using an anti-sense Oligo(dT) primer (dT12): 5'-pd(T)12(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'-AAGCTTATTTGATACAGTTTCC-3' and (RAB, for common reverse A/B sequence primer): 5'-AAGCTTATTTGATACAGTTTCC-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 Gsl 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'-GGGGCTATGGGTAAAAATGCTGTTCC-3' and (Bg-45) 5'-CTGAGCTCGACGACATTCA(TA)TACTGCT-3', for *TcPRACA* (codons +2 to +429) and (Hi-45) 5'-CTCTCCATGGGGCAGGGAGAAAAGCTTCTG-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 anti-sense 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 Xhol 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 Harirhan 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^ gene-ticin (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 TcPRAC fragment, Neo, gapdh intergenic region and rDNA gene spacer fragment-specific probes (Martinez-Calvillo et al., 1997) (see below).

**Western blots**

Recombinant *TcPRACA*, r*TcPRACB* (Reina-San-Martin 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.

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lose membranes. When indicated, total protein concentration of samples was adjusted to 300 μg ml⁻¹. Membranes were saturated in Tris-buffered saline and milk, washed, incubated with polyclonal serum against rTcPRAC, washed and developed with peroxidase-labelled secondary antibody using chemiluminescence (ECL kit, Amersham, Orsay, France). Blots were scanned and analysed with QUANTI 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 rTcPRAC followed by 4 μg 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 × 10⁷ 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 geneco, 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’-CGGGCTGACAGGCTATTC-3’) and neo-antisense (5’-AGGTAAGCAAGCAGTCG-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-²⁵P]-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 assessed in cultures of confluent monolayers of 2 × 10⁵ 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 × 10⁵ metacytic (or 10:1 ratio, whenever indicated) with WT or transgenic metacyclic parasites at 37°C, under 5% CO₂. 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 × 10⁴ ml⁻¹ and incubated with metacytic 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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**References**


Chamond, N., Gregoire, C., Coatnoan, N., Rougeot, C., Fre-


