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A B-cell mitogen from a pathogenic trypanosome is a eukaryotic proline racemase

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Lymphocyte polyclonal activation is a generalized mechanism of immune evasion among pathogens. In a mouse model of *Trypanosoma cruzi* infection (American trypanosomiasis), reduced levels of polyclonal lymphocyte responses correlate with resistance to infection and cardiopathy. We report here the characterization of a parasite protein with B-cell mitogenic properties in culture supernatants of infective forms, the cloning of the corresponding gene and the analysis of the biological properties of its product. We characterized the protein as a co-factor-independent proline racemase, and show that its expression as a cytoplasmic and/or membrane-associated protein is life-stage specific. Inhibition studies indicate that availability of the racemase active site is necessary for mitogenic activity. This is the first report to our knowledge of a eukaryotic amino acid racemase gene. Our findings have potential consequences for the development of new immune therapies and drug design against pathogens.

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The isolation and characterization of molecules essential in parasite metabolism or in the interactions of a parasite with host immune defenses are fundamental for the development of rational strategies for vaccination, immunotherapy and drug design. Attempts to provoke effective immunity to parasites are limited by poor specific immune responses to parasite antigenic molecules in early phases of infection[1], thus allowing immune evasion. Polyclonal lymphocyte activation is a general characteristic of infectious processes and the resulting nonspecific immune responses are associated with immunosuppression and autoimmunity, as seen in human and experimental infections by the protozoan parasite *Trypanosoma cruzi*, the etiological agent of Chagas disease[2–6]. The immunophysiopathology of this trypanosomiasis is manifested by cachexia, anemia and cardiac functional disorders, and esophageal and intestinal megasyndromes after lesions develop on the enervating plexus. Although the expansion of self-reactive B-cell clone populations and the hypergammaglobulinemia resulting from the 'panclonal' B-cell activity are believed to be at the origin of tissue damage, 'parasite evasion' results at least in part from the release of mitogenic or superantigenic moieties that inhibit host specific responses by triggering nonspecific lymphocyte activation[1–3]. Understanding the mechanisms underlying such polyclonal lymphocyte responses may indicate the way to neutralize it, and thus allow for effective immunity against infectious agents.

In the mouse model of *T. cruzi* infection, the abrogation or reduction of these polyclonal B- and T-cell responses leads to an increased resistance to infection and to the control of chronic tissue pathology[2,7–9]. Given that proteins released from trypomastigote forms of *T. cruzi* behave as polyclonal B-cell activators[10], infective metacyclic forms might show an increased production of mitogenic molecules, thereby promoting such a mechanism of immune evasion.

We used biochemical and subsequent molecular approaches to isolate from culture supernatants of metacyclic trypomastigotes the parasite molecules involved in the induction of such polyclonal B-cell responses. Here we present the identification and molecular characterization of a *T. cruzi* gene, and demonstrate *in vivo* and *in vitro* that its product is a B-cell mitogen. In addition we show that this protein is in fact a eukaryotic proline racemase whose activity seems to be essential for B-cell mitogenicity. Given the increasing interest in the participation of D-amino acids in biological processes and immune phenomena, our findings provide new information on the involvement of parasite amino acid racemases in pathogen–host interactions, indicating that they may serve as targets for the induction of protective immunity and therapy.

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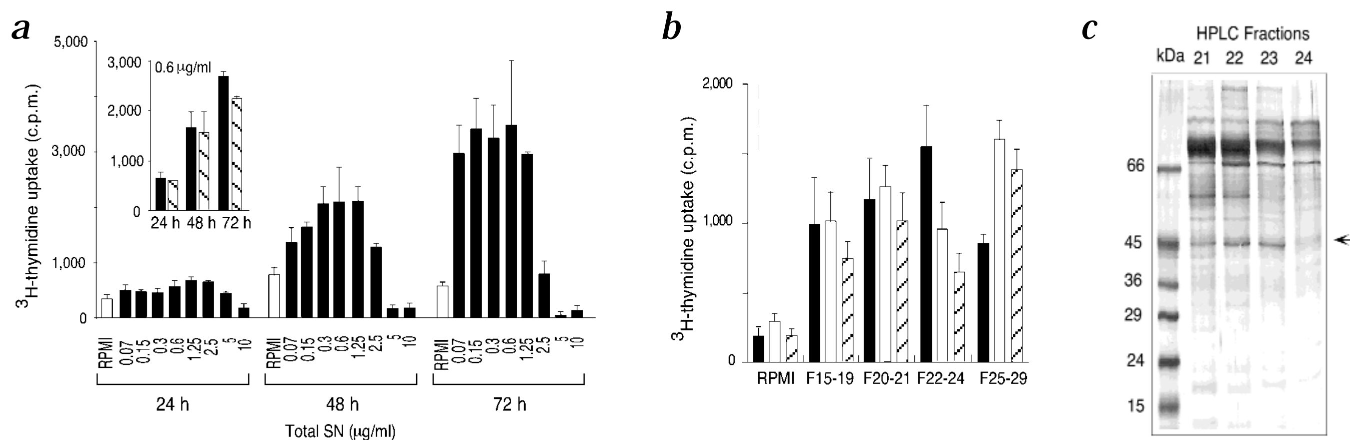

Fig. 1 A 45-kDa B-cell polyclonal activator isolated from parasite culture supernatants. ***a***, Proliferative activity of total spleen cells stimulated *in vitro* by increasing concentrations (below graph) of total proteins from culture supernatants of metacyclic trypomastigotes differentiated *in vitro*. Inset, Proliferation of total (■) or T cell-depleted (◩) splenocytes in the presence of proteins from total culture supernatant. c.p.m., counts per minute ***b***, Proliferative activity of total spleen cells stimulated *in vitro* by HPLC pooled fractions at 24 h (■), 48 h (□) and 72 h (◩). ^{3}H-thymidine uptake (c.p.m., counts per minute) after 48 h in the presence of concavalin A and lipopolysaccharide was 32,000 and 3,500 counts per minute, respectively. To rule out the possibility of a mitogenic effect due to lipopolysaccharide contamination in the samples, proliferation assays were also done using freshly recovered splenocytes from the 'lipopolysaccharide non-responder' mouse strain C3H/He; the same levels of proliferation were obtained. ***c***, 8% SDS–PAGE analysis of fractions 21–24 (silver staining). Left margin, molecular size markers. Arrow, main candidate.

Isolation and cloning of a *T. cruzi* 45-kDa B-cell mitogen

To investigate the release of proteins with B-cell mitogenic activity by *T. cruzi*, we produced culture supernatants of *in vitro*-differentiated metacyclic trypomastigotes in a protein-free defined medium[11]. We confirmed lymphocyte mitogenic activity in total concentrated culture supernatants by lymphocyte proliferation assays. Proliferation was sustained over a 72-hour period of culture in a dose-dependent manner (Fig. 1*a*). As with other B-cell mitogens, we found the same level of proliferation when we tested supernatants on T cell-depleted splenocytes (Fig. 1*a*).

To identify the molecules responsible for mitogenic activity, we fractionated the parasite proteins present in the metacyclic culture supernatants by high-performance liquid chromatography (HPLC)/anion-exchanger chromatography, and tested the resulting fractions as described above. Fractions 22–24 (eluted at an ammonium acetate concentration between 368 and 467 mM) showed the highest ^{3}H-thymidine uptake at 24 hours, decreasing thereafter (Fig. 1*b*). This 'precocious' peak followed by an early decrease in cell proliferation, not obtained with the other fractions, could be indicative of a higher concentration of the molecule of interest after purification promptly triggering cell activation, or (less likely) the presence of toxic products present only in fractions 22–24. Experiments using parasite culture supernatants purified by DEAE chromatography have shown that a protein fraction 40–45 kilodaltons (kDa) in molecular mass is able to induce B-cell activation and proliferation *in vivo*. (B.R.-S.M. *et al.*, unpublished data) SDS–PAGE analysis of the HPLC fractions showed the presence of a protein with an apparent molecular mass of 45 kDa only in fractions 21–24 (Fig. 1*c*). We estimated the isoelectric point of the protein to be between 4.5 and 5.0 by isoelectric focusing (data not shown). Thus, we chose the 45-kDa protein band as the main candidate (Fig. 4c, arrow).

To obtain peptide sequences from the 45-kDa protein (called Tc45 here) and to permit subsequent PCR-assisted cloning of a gene fragment, we isolated the 45-kDa protein band by SDS–PAGE and undertook its internal digestion and microsequencing. We isolated and sequenced six peptides (uncertain amino acid identification, in parenthesis): (W)IIK (residues 43–46), IVTGSLP(D)I(S)G (residues 90–100), ATNVPVVLDTPAGLVR (residues 183–198), VDIAFGGNF (residues 241–249), NVVIFGNR (residues 316–323) and MATLYAK (residues 338–344)(Fig. 2*a*, underlining). We used peptides 4 and 5 for degenerate primer design based on the relatively low level of degeneracy in their corresponding coding sequences (Fig. 2*a*, italics). We reverse-transcribed total RNA from *T. cruzi* trypomastigote forms using reverse degenerate primers for both peptides. We then used the resulting cDNA as a template for PCR amplification. From all possible combinations, when the PCR reaction comprised forward primer for peptide 4, reverse primer for peptide 5 and template cDNA synthesized with reverse primer for peptide 5, only one PCR product 239 base pairs (bp) in length contained both primers, after cloning and sequencing. The fragment contained an unique open reading frame (ORF) flanked by peptides 4 and 5 coded in-frame.

Characterization of the Tc45 gene

To obtain the full sequence of the *Tc45* gene, we used the ^{32}P-labeled 239-bp PCR product as a probe to screen a *T. cruzi* clone CL-Brener lambda Fix II genomic library. We isolated four independent positive phages. Restriction analysis and Southern blot hybridization showed two types of patterns, each represented by two phages, indicating that the *Tc45* gene is present in at least two copies per haploid genome. The complete sequence of the *Tc45-A* gene and flanking regions from one of the phages showed an ORF of 423 codons containing all sequenced peptides (Fig. 2*a*). Computer analysis predicted a 29-amino-acid signal peptide (Fig. 2*a*, double-headed arrow), indicating active secretion by *T. cruzi*, which is in agreement with the fact that the protein was purified from culture supernatants. There were a

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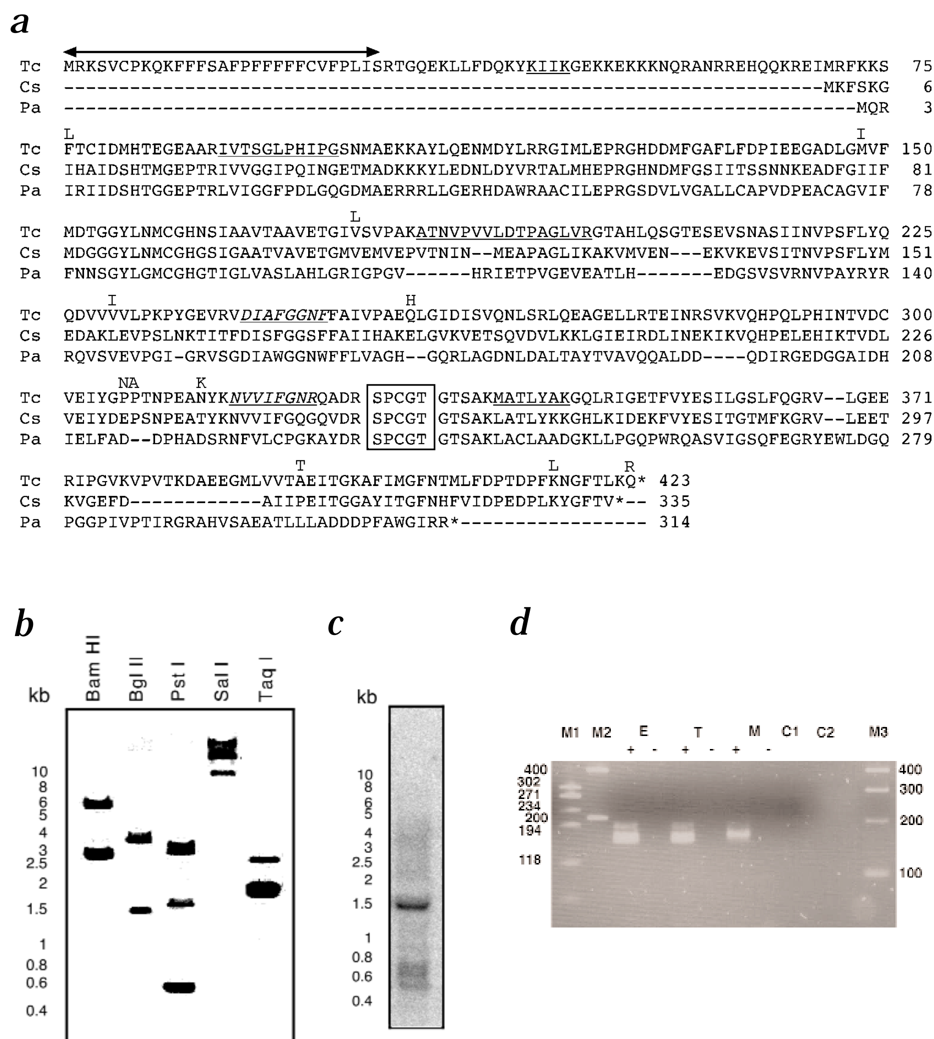

Fig. 2 Homology between the Tc45 protein and the bacterial proline racemases, and the genomic organization and transcription of the *Tc45* gene. ***a***, Protein sequence alignment of *T. cruzi* Tc45-A (Tc), *C. sticklandii* (Cs) and *P. aeruginosa* (Pa) proline racemases. Double-headed arrow, computer-predicted signal peptide; underlining, peptide sequences obtained by microsequencing; italics, peptides used for designing degenerate primers; box, proline racemase active site; dashes, gaps generated for best fit; single letters above the Tc45-A sequence, point mutations in the predicted Tc45-B protein sequence already identified. ***b***, Southern blot analysis of *T. cruzi* genomic DNA (restriction enzymes, above blot) hybridized with a ^{32}P-labeled probe including the TcPA45 coding sequence. Left margin, molecular sizes in kilobases (kb). ***c***, Northern blot analysis of total RNA from epimastigote forms hybridized with a ^{32}P-labeled probe as described above. Left margin, molecular sizes in kilobases (kb). ***d***, mRNA expression of TcPA45 in different life stages of the parasite, shown by electrophoresis of gene fragments obtained by specific reverse transcription from total parasite RNA followed by PCR amplification using the sequences of the mini-exon (spliced leader) and R-300-45 primers and subsequent amplification of a 170-bp internal fragment. M1, M2 and M3, molecular size markers (sizes, left and right margins). First-strand cDNA reactions were done in the presence (+) or absence (–) of reverse transcriptase, to exclude the possibility of further PCR amplification of fragments due to genomic DNA contamination. C1 and C2, internal negative (no template) controls; E, epimastigote; T, trypomastigote; M, metacyclic.

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poly-pyrimidine rich region and a probable trans-splice acceptor site 56 and 7 bp, respectively, upstream of the ATG codon. An alternative trans-splicing signal was present about 170 bp upstream of the second ATG codon within the coding region that, if used, would allow the expression of a truncated, non-secreted protein lacking 69 amino acids. Polyadenylation may take place at positions 1,442 or 1,443, which were preceded by repeats of the triplet UUA, a motif found at the 3′ end of other *T. cruzi* genes[12,13].

To investigate the genomic organization and transcription of the *Tc45* gene, we used Southern blot analysis; this indicated the presence of two gene copies per haploid genome. There are probably two homologous *Tc45* genes (Fig.2*b*). Digestion with *Bam*HI and *Bgl*II produced two hybridizing bands, consistent with the presence of two gene copies, as the probe has neither enzyme restriction site. High-molecular-weight DNA hybridized after probable partial digestion with *Sal*I, consistent with the absence of this site within the coding sequence covered by the probe. Both *Pst*I and *Taq*I cleaved within the probe and produced more than one hybridizing band per gene copy. Preliminary results indicated that they are located on different chromosomes (data not shown). Northern blot analysis on total RNA from epimastigotes showed a transcript of around 1.5 kb, as expected from the genomic sequence (Fig. 2*c*). We confirmed the presence of the Tc45 mRNA in different parasite forms by reverse transcription PCR using primers specific for the *Tc45* gene (Fig. 2*d*). Several point mutations were already identified in the sequence of the putative *Tc45-B* gene copy representative of the second phage type (Fig. 2*a*), and further transcriptional and functional analyses of the alleles are underway.

To identify homologies with other genes, we compared the *Tc45-A* gene copy and protein sequence to several databases. There was homology (nucleotide sequence, 57.7% identical; amino-acid sequence, 52.4% identical) with the only proline racemase described[14], an intracellular homodimeric protein isolated from *Clostridium sticklandii*. This enzyme catalyzes the interconversion between the L- and D- proline enantiomers, and its reaction mechanism has been studied extensively[14]. There was also homology with the translation of an ORF sequence from *Pseudomonas aeruginosa* in contig 53 of the unfinished Pseudomonas genome project (amino-acid sequence, 37.9% identical; *C. sticklandii* proline racemase and the translation of

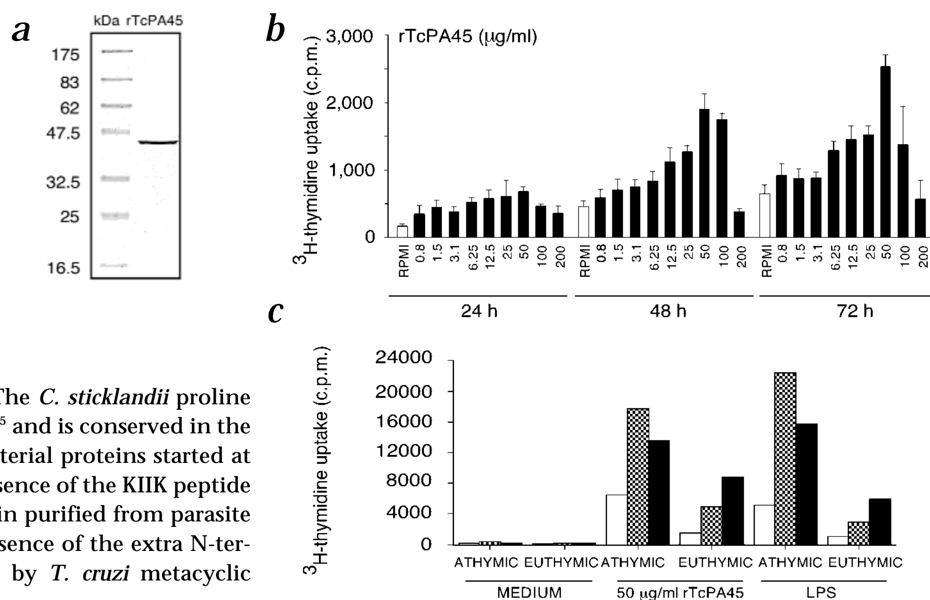

Fig. 3 Characterization of rTcPA45 mitogenic activity. ***a***, 8% SDS–PAGE analysis of rTcPA45 (Coomasie blue staining). Left margin, molecular sizes. ***b***, Proliferative activity of total splenocytes in the presence of increasing concentrations (below graph) of rTcPA45. ***c***, Proliferative activity of total splenocytes obtained from athymic or euthymic mice in the presence of rTcPA45 or lipopolysaccharide, at 24 h (□), 48 h (■) or 72 h (■).

the *P. aeruginosa* ORF, 47% identical). The *C. sticklandii* proline racemase active site has been identified[15] and is conserved in the Tc45 protein. Homology with both bacterial proteins started at amino acid 70 of Tc45 (Fig. 2*a*). The presence of the KIIK peptide (Fig. 2*a*, underlining) in the Tc45 protein purified from parasite culture supernatants confirmed the presence of the extra N-terminal portion of the protein released by *T. cruzi* metacyclic forms.

Tc45 is a B-cell mitogen and a eucaryotic proline racemase

To demonstrate the mitogenic and proline racemase activities of the Tc45 protein, we overexpressed in *Escherichia coli* and purified a recombinant protein "tagged" at the C terminus with six histidines (rTc45, starting at codon 30 and thus excluding the predicted signal peptide; Fig. 3*a*). By *in vitro* proliferation assays of naive murine spleen cells, recombinant protein rTc45 had mitogenic activity similar to that of the native protein fraction purified from culture supernatants. Thus, rTcPA45 (for the *T. cruzi* polyclonal activator of 45 kDa) induced spleen lymphocyte proliferation that increased with time over 72 hours of culture (Fig. 3*b*). Proliferation was dose dependent, with a bell-shaped response curve (starting at 0.8 µg/ml and peaking at 50 µg/ml), typical of all mitogens described so far. rTcPA45 is indeed a T cell-independent polyclonal activator of B lymphocytes, as shown by the magnitude and increase of the proliferative response of total spleen lymphocytes obtained from athymic mice compared with the response of lymphocytes from euthymic individuals (Fig. 3*c*). Injection of 50 µg rTcPA45 *in vivo* induced an increase of 200% in spleen cell numbers by day 4, accompanied by an increase of 250–1,00% in numbers of immunoglobulin (Ig)-secreting B cells of the IgM, IgG2a, IgG2b and IgG3 isotypes, while showing a complete lack of rTcPA45-specific Ig-secreting B cells, indicating the polyclonal B-cell mitogenicity of the protein (data not shown).

To demonstrate that the TcPA45 protein is indeed a proline racemase, we used *in vitro* biochemical assays to measure the shift in optical rotation of either L- or D-proline substrates. rTcPA45 racemized both L- and D- proline but not L-hydroxyproline or any other natural L-amino acids (Fig. 4*a*). Such rTcPA45 racemase activity was co-factor-independent, notably of pyridoxal phosphate, and thus closely resembles the *C. sticklandii* proline racemase[14]. Furthermore, the rTcPA45 enzymatic activity was inhibited to different extents by the presence of inhibitors[14] such as maleic acid, iodoacetamide, iodoacetate and, most particularly, pyrrole-2-carboxylic acid, a potent and specific inhibitor of proline racemase (Fig. 4*b*). Indeed, this molecule is planar and most probably binds more tightly to the substrate binding site[14]. The proline racemase activity of rTcPA45 was greatest at a pH of 6 (Fig. 4*c*), which is two units lower than that of the bacterial enzyme[14]. The optimal temperature for enzymatic activity was 37 °C and the enzyme was inactivated after being heated for 10 minutes at 80 °C (data not shown). This is the first description to our knowledge of an amino-acid racemase gene in an eukaryotic organism.

Cellular localization of TcPA45

To analyze the cellular localization of the parasite TcPA45, we used immunofluorescence experiments with a polyclonal serum raised against rTcPA45. Whereas serum from chronically infected mice stained trypomastigote cells uniformly, rTCPA45-specific antibodies stained mostly the cytoplasm of epimastigote forms but not the nucleus or the kinetoplast (Fig. 5*a*). *In vitro*-differentiated metacyclic forms showed a less-intense and more-diffuse pattern of cytoplasmic staining than did epimastigotes. However, bloodstream trypomastigote forms were strongly labeled at the flagellar pocket and the anterior and posterior ends of the parasite, and lightly along the flagellum and cytoplasm. These experiments substantiate the hypothesis that *T. cruzi* has an intracellular form of the proline racemase and that secretion might only take place in the infective forms. Western blot analysis of cell extracts of the parasite confirmed that the TcPA45 protein was present in different developmental stages (Fig. 5*b*). We detected a TcPA45 protein around 39 kDa in molecular mass in epimastigotes (non-infective insect forms) and 41.5 kDa in infective metacyclic trypomastigotes, compared with the computer-predicted molecular masses of 43.4 kDa and 38 kDa, respectively, for a secreted and a non-secreted form of the protein (Fig. 5*c*). Western blot analysis of the non-infective epimastigote cell stage showed Tc45 proline racemase mostly in the soluble cellular fraction, only weakly in the cellular insoluble fraction and absent from culture medium (Fig. 5*d*).

TcPA45 mitogenic and racemase activities are linked

To confirm the relationship, if any, between the enzymatic and the mitogenic activities of rTcPA45, we used *in vitro* proliferation assays with active rTcPA45 and different forms of the inactivated enzyme. Unexpectedly, mitogenic activity was abolished when

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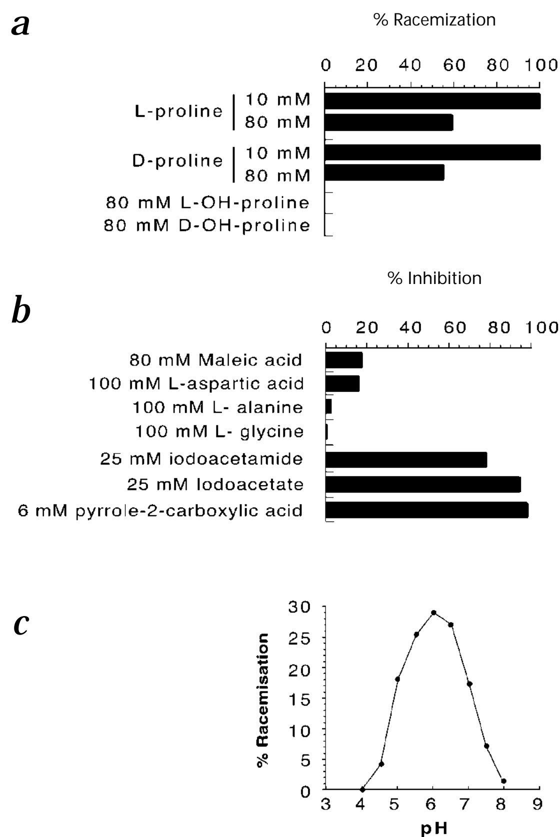

Fig. 4 rTcPA45 is a proline racemase. ***a***, Percent racemization of L-proline, D-proline, L-hydroxy (OH)-proline and D-hydroxy (OH)-proline substrates. ***b***, Percent inhibition of racemization of 80 mM L-proline in the presence of inhibitors (left margin). ***c***, Percent racemization of 80 mM L-proline as a function of pH.

rTcPA45 was inactivated by being heated or by long storage at 4 °C, or whenever enzymatic inhibition was achieved by pre-incubation of the protein with specific (pyrrole-2-carboxylic acid) or nonspecific (iodoacetamide and iodoacetate) inhibitors of proline racemase (Fig. 6*a*). Mitogenic activity was also affected considerably by supplementation of the cultures with increasing amounts of L- or D- proline, in a dose-dependent manner, indicating that competitive inhibition occurred in the presence of specific substrates (Fig. 6*b*). There was no cell proliferation when lymphocytes were cultured in the presence of 50 mM L- or D-proline alone (Fig. 6*b*). Furthermore, mitogenic activity due to another B-cell mitogen, antibody against mouse IgM (μ chain), was unaffected by the same inhibitors or substrates (data not shown). Although the antibodies against TcPA45 raised against the recombinant protein were not able to inhibit racemization or to neutralize mitogenic activity *in vitro*, and thus cannot be used to support a link between these activities, our results indicate that a free and intact active site of the rTcPA45 protein is necessary to allow mitogenicity.

Discussion

Here we have shown the biochemical isolation, cloning and molecular characterization of a B-cell mitogen released by *T. cruzi*. Unexpectedly, we have also described a racemase in a parasite, raising questions about *T. cruzi* biology and its interaction with the mammalian host. What would be the function of such enzymatic activity in the biology of *T. cruzi?* In bacteria, amino-acid racemases are cytoplasmic proteins that participate in metabolic processes or in the synthesis of peptides modified post-translationally[16]. *T. cruzi* can use L-proline as a principal source of carbon[17], possibly through a D-proline intermediate[18]. Preliminary results using defined culture media indicate that both epimatigote and metacyclic trypomastigotes can metabolize both L- or D-proline as sole carbon source, indicating that proline racemase is important in the energy metabolism of the parasite.

Proteins with D-amino acids are very resistant to eukaryotic proteases. Thus, the parasite may also use the racemization mechanism to synthesize and express on its surface proteins containing D-proline, thereby ensuring a certain degree of resistance to host-induced proteolytic mechanisms. Additionally, proline racemase activity of the TcPA45 protein might be involved in the differentiation of *T. cruzi* from epimastigote to trypomastigote, as this process is induced in the presence of L-proline at a pH of 6 in the insect's gut[19] as well as during *in vitro* metacyclogenesis[11].

TcPA45 released by metacyclic infective forms seems to be directly mitogenic for B lymphocytes, as indicated by the *in vitro* and *in vivo* results, as mitogenic activity relies on the intact enzymatic activity of TcPA45. Possibly the conformational structure of active TcPA45 itself permits its binding to B-cell expressed (perhaps proline-rich) motifs, thus triggering its activation. Therefore, the apparent direct B-cell mitogenicity of TcPA45 was considerably compromised when specific inhibitors of the proline racemase active site were used (pyrrole-2-carboxylic acid), or through substrate competition. However, TcPA45 itself might not be mitogenic, but may act through racemization or by binding to host molecules, which would be the primary mitogens. Regardless, nonspecific lymphocyte activation would ensure evasion of the parasite at the very beginning of infection.

There are additional possible functions for parasite secretion of TcPA45. TcPA45 may be involved in the modification of membrane molecules of the host target cells facilitating adhesion and/or subsequent parasite penetration and multiplication. Furthermore, involvement of epimerases, growth factors and mitogens has been indicated in the reactions leading to the attachment of heparan sulfate chains to core macromolecules of cardiovascular cells[20], one of the targets of *T. cruzi* invasion. It is perhaps relevant that invasion of host cells by *T. cruzi* relies on heparan sulfate receptors[21], heparin and collagen, three components of the extracellular matrix[22], in a way analogous to the function of the circumsporozoite protein of Plasmodium sporozoites that use heparan sulfate proteoglycans as receptors for adhesion and invasion of host liver cells[23]. Experiments are underway to test this.

Our results using immunofluorescence and western blot analyses indicated that *T. cruzi* can differentially express intracellular and secreted forms of the TcPA45 protein. Indeed, the *TcPA45-A* gene codes for an extra N-terminal 70 amino acids predicted to contain a signal peptide (compared with the *C. sticklandii* proline racemase sequence), and an alternative trans-splicing signal is present within the coding sequence. Alternatively, initiation of translation might occur at the first or second ATG codon after the splice leader sequence, depending on the need for an intracellular or extracellular TcPA45, or the two gene copies might be differentially expressed. Any of these mechanisms would ensure that infective trypomastigotes ac-

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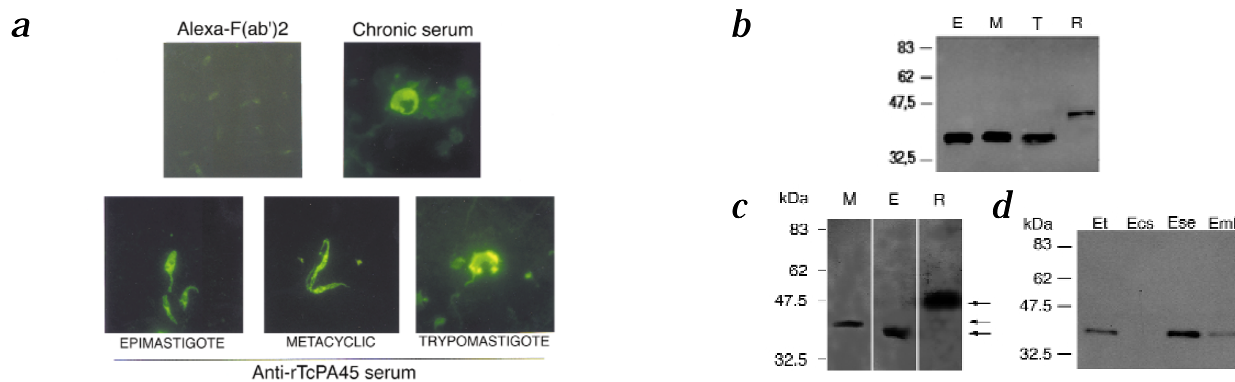

Fig. 5 Differential expression of rTcPA45 protein in the parasite. ***a***, Cellular localization of the Tc45 protein in different life stages of the parasite, shown by indirect immunofluorescence using polyclonal mouse serum against rTcPA45 followed by staining with the Alexa 488™ goat antibody against mouse IgG (H+L), $F(ab')_2$ fragment conjugate (bottom row), compared with control staining using the Alexa 488™ $F(ab')_2$ fragment conjugate alone (top left) or after incubation of the parasites with serum from chronically infected mice (Chronic serum). ***b*** and ***c***, Detection of Tc45 protein in total extracts of epimastigote (E), metacyclic (M) and trypomastigote (T) forms of the parasite, compared with recombinant rTcPA45 (R) protein, by western blot analysis. Arrows, calculated molecular weights for isoforms of the TcPA45 (R) protein. ***d***, Presence of the 39-kDa isoform of the Tc45 protein in total (Et), soluble (Ese) and insoluble (Emb) sonic extracts of non-infective epimastigote forms of the parasite, compared with its absence in culture supernatants (Ecs). Molecular sizes (*b*–*d*), left margins.

quire the ability to release a form of the protein involved in the interaction with host cells, including B-cell triggering. Full transcriptional analysis and the genetic disruption of this gene is now in progress and will allow us to test different hypotheses. The disruption of alanine racemase and the D-amino acid aminotransferase genes of *Listeria monocytogenes* results in the inability of the bacteria to grow within the eukaryotic host cells[24]. Both of these gene products are involved in the synthesis of D-alanine that is required for the production of a mucopeptide component of the cell walls of almost all bacteria. It remains to be determined whether such bacterial molecules are B-cell mitogens as predicted. Accordingly, work with random amino-acid polymers has established that multichain polypeptides composed of D-amino acids induce antibody responses in a T cell-independent manner[25,26], a property that can be interpreted as equivalent to B-cell mitogenicity[27,28]. Resistance of such D-amino-acid peptides to degradation by host enzymes[29] could also explain the persistence of polyclonal responses (and immunosuppression)[30], even if parasite production of the mitogen would be transient at the start of infection.

So far, there is no effective treatment or vaccine against *T. cruzi* infection and Chagas disease pathology. Attempts to isolate immunodominant protective epitopes have failed[1]. As indicated[1,6], and as has been demonstrated for *Candida albicans* infections[31], mitogenic moieties can be used as vaccination targets to induce specific neutralization of the mitogen, thus aborting the parasite 'strategy' to deviate immune responses into a nonspecific polyclonal activation and immunosuppression. The molecule described here could represent an appropriate target for such attempts. Indeed, our preliminary results indicate that intramuscular DNA vaccination protocols using pcDNA3 vector containing the *TcPA45* gene, with or without the fragment encoding the signal peptide, are able to induce a decrease of 85% in parasitemia levels after challenge with infective forms of the parasite. Moreover, there were even higher levels of parasitemia control when sub-mitogenic doses of the active rTcPA45 protein were injected intraperitoneally 2 weeks before infective challenge. These observations support the potential use of this molecule as a drug and/or immunomodulator target.

Finally, there is a growing interest in the biological function

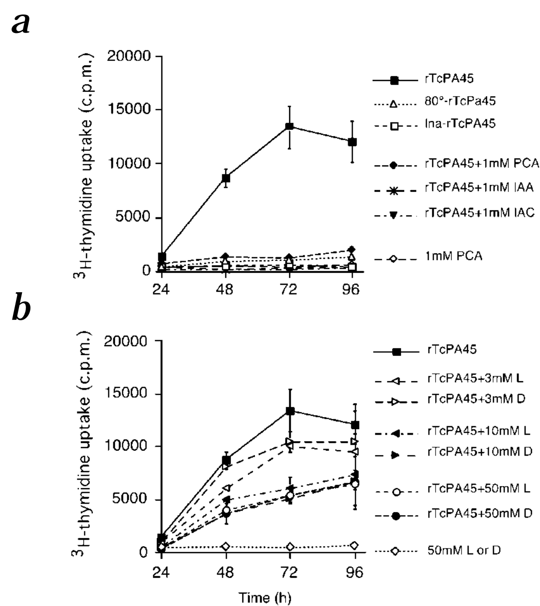

Fig. 6 Correlation between mitogenic and racemase activities. ***a***, Proliferative activity of total mouse splenocytes in the presence of rTcPA45 protein that is enzymatically active (rTcPA45) or lacking racemase activity by being heated (80°-rTcPA45), by long term storage at 4 °C (Ina-rTcPA45), or by pre-incubation of rTcPA45 with pyrrole-2-carboxylic acid (rTcPA45 + 1 mM PCA), iodoacetamide (rTcPA45 + 1 mM IAA) or iodoacetate (rTcPA45 + 1 mM IAC) inhibitors, compared with 1 mM pyrrole-2-carboxylic acid (1mMPCA) alone. ***b***, Competitive inhibition of rTcPA45-induced proliferative activity of total mouse lymphocytes by increasing concentrations of L- or D-proline substrates. Controls, cultures of splenocytes with 50 mM L- or D- proline alone. c.p.m., counts per minute.

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of D-amino acids, either as free molecules or within polypeptide chains in human brain, tumors, antimicrobial peptides and neuropeptides, as well as in 'protein fatigue'[32], indicating widespread biological consequences. Research on D-amino acids in living organisms has been hampered by the difficulty in detecting them. However, recent purification of a serine racemase from mammalian brain[33] indicates conservation throughout evolution. Our work may stimulate further research on the biological function of such enzymes and their biochemical and biological consequences in immune phenomena.

Methods

Mice and parasites. Male euthymic or athymic BALB/c mice 8 weeks of age were purchased from Charles River Laboratories (Saint Aubin les Elbeuf, France). Male C3H/HeJ mice 8 week of age from our animal facilities were also used. *T. cruzi* (clone CL Brener) was used throughout. Epimastigotes were maintained by weekly passage in liver infusion tryptose medium. *In vitro* metacyclogenesis was accomplished in a protein-free defined medium at 28 °C, as described[11]. Trypomastigote forms were obtained from the blood of C3H/HeJ mice used for serial passages of the parasite *in vivo*.

Protein fractionation. Culture supernatants 40 l in volume from metacyclic forms, maintained for an additional 96 h at 37 °C, were concentrated by vacuum dialysis and dialyzed against buffer A. HPLC used the weak anion exchanger column POROS HQ-10 (Perspective Biosystems, Framingham, Massachusetts) at a flow rate of 1 ml/min according to the following program: 10 min with buffer A, 30 min linear gradient from buffer A to B, 5 min linear gradient from buffer B to C, and 5 min with buffer C (buffers: A, 5 mM ammonium acetate, pH 8; B, 1 M ammonium acetate, pH 8; C, 1 M NaCl and 1 M ammonium acetate, pH 8). Fractions 1 ml in volume were collected, frozen at –80° C, lyophilized and reconstituted in water or in non-supplemented RPMI medium for *in vitro* proliferation assays. SDS–PAGE analysis used standard techniques.

Generation of peptides and amino-acid sequence analysis. HPLC fractions 22 and 23 were pooled and separated by 8% SDS–PAGE. After amido black staining, a protein band of about 45 kDa was cut out, 'in-gel digested' with trypsin and submitted to reverse-phase HPLC to separate peptides. Automated Edman degradation sequence analysis was done in the Laboratoire de Microséquençage de Protéines of the Pasteur Institute.

RNA preparation, reverse transcription, PCR and cloning. RNA was extracted from trypomastigote forms obtained from Vero cells, using TRIzol LS reagent (Life Technologies) following the manufacturers' instructions. Of this RNA, 2 µg were reverse-transcribed in 20 µl with Superscript II (Life Technologies) using the antisense degenerate primer 5′–TTICCRAADATIACIACGTT–3′ designed from peptide 5. DNA was amplified by PCR using 5 µl cDNA and Taq polymerase (Perkin Elmer, Montigny-le Bretonneux, France) or Pfu DNA polymerase (Stratagene, Saint Quentin en Yvelines, France). PCR conditions for amplification of the *TcPA45* gene fragment (239 bp) comprised 30 cycles of 30 s at 94 °C, 45 s at 45 °C and 30 s at 72 °C, followed by 10 min at 72 °C. Degenerate primers were 5′–ATHGCITTYGGIGGIAAYTTT–3′ and 5′–TTICCRAADATIACIACGTT–3′ (peptides 4 (forward) and 5 (reverse), respectively). Here, D indicates A, G or T; H indicates A, C or T; M indicates A or C; I indicates inosine; R indicates A or G; and Y indicates C or T. PCR conditions for amplification of the *TcPA45* coding sequence (codons 30–423) comprised 20 cycles of 45 s at 94 °C, 45 s at 50 °C and 3 min at 72 °C, with primers 5′–CTCTCCCATGGGGCAGGAAAAGCTTCTG–3′ and 5′–CTGAGCTCGACCAGATCTATCTGC–3′. PCR products were purified with the Qiagen PCR extraction kit (Qiagen, Courtaboeuf, France) and cloned into pCR II-TOPO vector using the TOPO-TA cloning kit (Invitrogen, Groningen, The Netherlands) following the manufacturer's instructions.

Automated sequencing. Lambda phage and plasmid DNA were prepared using standard techniques, and direct sequencing was accomplished with the Big Dye Terminator kit (Perkin Elmer, Montigny-le Bretonneux, France) following the manufacturer's instructions. Extension products were run for 7 h in an ABI 373B automated sequencer. Primers internal to the sequence have also been used for sequencing.

Genomic library screening. A genomic library of *T. cruzi* CL-Brener constructed in phage lambda Fix II (provided by E. Rondinelli) was screened using a ^{32}P-labeled 239-bp PCR product as a probe. Hybridization used standard conditions. Filters were scanned using a PhosphorImager scanning unit (Molecular Dynamics, Ivry, France). Positive phages were identified and phage DNA was prepared using standard procedures.

Expression constructs and recombinant protein expression. The PCR product encoding the *TcPA45* gene fragment starting at codon 30 was cloned in frame with a C-terminal six-histidine tag into the pET28b(+) expression vector (Novagen-Tebu, Le Parrayen Yvelines, France). Soluble recombinant protein was produced in *E. coli* and purified using a nickel column (Novagen-Tebu, Le Parrayen Yvelines, France) following the manufacturer's instructions.

Assays of proliferative activity. *In vitro* proliferation was accomplished using freshly recovered splenocytes from BALB/c mice seeded at a density of 5×10^4 cells/well and incubated for 24, 48 and 72 h with increasing concentrations of total parasite supernatants or recombinant TcPA45 protein (0.07–200 µg/ml), with 0.5 µg/ml of the HPLC fractions, or with the conventionally used mitogens concanavalin A (10 µg/ml) and lipopolysaccharide (5 µg/ml) in 5% FCS in RPMI-1640 complete medium. T-cell depletion was accomplished by incubating freshly recovered spleen cells for 30 min at 37 °C in the presence of monoclonal antibodies against Thy 1.2 and rabbit complement (Cedarlane, Le Perray en Yvelines, France). Analysis of proliferative activity of total splenocytes (5×10^4 cells/well) in the presence of 50 µg/ml enzymatically active rTcPA45 was also compared with the proliferation obtained using the same amounts of rTcPA45 protein lacking racemase activity (by heating for 10 min at 80 °C or by long term storage at 4 °C). Inhibition of proliferation was obtained by adding to the splenocyte cultures 50 µg/ml rTcPA45 pre-incubated for 10 min at 37 °C with 1 mM inhibitor, either specific (pyrrole-2-carboxylic acid) or nonspecific (iodoacetamide or iodoacetate). Competitive assays of proliferative activity by 50 µg/ml rTcPA45 were done by adding increasing concentrations of specific proline racemase substrates (L- or D- proline) ranging from 3 mM to 50 mM. Controls included the incubation of splenocytes (5×10^4 cells/well) with 50 mM L- or D- proline in RPMI medium alone. Cultures were collected after a 16-hour pulse of 1 µCi/well ^{3}H-thymidine. ^{3}H-thymidine uptake was determined in a beta-plate liquid scintillation counter (LKB-Wallac, Orsay, France). All data points were obtained in triplicate and the corresponding standard deviation was calculated.

Southern, western and northern blots. Mice were immunized intrasplenically with 10 ng protein and were boosted every 3 weeks with 1 µg of the same preparation for 2 months to obtain polyclonal serum containing rTcPA45-specific antibodies. Total, soluble and insoluble sonic extracts, or culture supernatants from the different parasite forms were purified and separated by 8–10% SDS–PAGE, and proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were saturated with Tris-buffered saline and milk, incubated with polyclonal serum against rTcPA45 and developed with peroxidase-labeled secondary antibody using an ECL kit (Amersham, Orsay, France). *T. cruzi* genomic DNA (10 µg) was digested with restriction enzymes (*Bam*HI, *Bgl*II, *Sal*I, *Taq*I and *Pst*I), separated by 0.8% agarose gel electrophoresis and transferred to Hybond N+ followed by hybridization of the membrane with a ^{32}P-dATP-labeled probe covering the TcPA45-coding sequence. Total RNA was prepared from epimastigote, metacyclic and trypomastigote forms of the parasite by conventional methods. For northern blot analysis, 20 µg epimastigote RNA was transferred to Hybond N+ membranes, then hybridized with single-stranded DNA complementary to the *TcPA45* gene transcript, labeled with α-^{32}P-dCTP.

Transcript analysis through reverse transcription and PCR. Total RNA (1 µg) from epimastigote, metacyclic and trypomastigote forms of the parasite were used to synthesize specific first-strand cDNA by using oligonucleotide R300-45 (5′–TCCGTATGCATGTCGATGC–3′), located about 240 nucleotides downstream from the first ATG start codon, fol-

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lowed by PCR amplification using R300-45 and an oligonucleotide corresponding to part of the *T. cruzi* spliced leader sequence (5′–TATTATTGATACAGTTTCTG–3′). An internal TcPA45 fragment of about 170 bp was then amplified using R300-45 and the oligonucleotide HI-45 (5′–CTCTCCCATGGGGCAGGAAAAGCTTCTG–3′) to demonstrate the presence of Tc45 transcript in each of the life stages analyzed.

Immunofluorescence. Cellular localization of TcPA45 protein in epimastigote, metacyclic and bloodstream forms of the parasite was demonstrated by indirect immunofluorescence using polyclonal mouse serum against rTcPA45 (described above) followed by 4 μg/ml Alexa 488™ goat antibody against mouse IgG (H+L), $F(ab')_2$ fragment conjugate (Molecular Probes-Interchim, Montluçon, France), compared with control staining using Alexa 488™ $F(ab')_2$ fragment conjugate alone or after incubation of the parasites with chronic serum obtained from mice infected for 8 months.

Racemisation assays. The percent of racemization of different concentrations of L-proline, D-proline, L-hydroxy-proline and D-hydroxy-proline substrates was calculated by incubating a 500-μl mixture of 3 μg rTcPA45 and 10–80 mM substrate in 0.2 M sodium acetate and 25 mM β-mercaptoethanol, pH 6, for 30 min at 37 °C. The reaction was stopped by incubation for 10 min at 80 °C. Water (1 ml) was then added, and the optical rotation was measured in a polarimeter 241 MC (Perkin Elmer, Montigny-le Bretonneux, France) at a wavelength of 365 nm, in a cell with a path length of 10 cm. The percent inhibition of racemization of 80 mM L-proline was determined in the presence of different concentrations of several specific and nonspecific inhibitors ranging in concentration from 6 mM to 100 mM. The percent racemization of 80 mM L-proline as a function of pH was determined using 0.2 M sodium actetate, potassium phosphate and Tris–HCl buffers containing 25 mM β-mercaptoethanol; reactions were incubated for 30 min at 37 °C as described above. All reagents and inhibitors were purchased from Sigma.

Accession numbers. The GenBank accession number of *T. cruzi TcPA45* is AF195522. The EMBL accession number of *C. sticklandii* is E10199

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