Biochemical Characterization of Proline Racemases from the Human Protozoan Parasite Trypanosoma cruzi and Definition of Putative Protein Signatures*

Received for publication, October 23, 2002, and in revised form, January 30, 2003
Published, JBC Papers in Press, February 11, 2003, DOI 10.1074/jbc.M210830200

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Proline racemase catalyzes the interconversion of L- and D-proline enantiomers and has to date been described in only two species. Originally found in the bacterium Clostridium sticklandii, it contains cysteine residues in the active site and does not require co-factors or other known coenzymes. We recently described the first eukaryotic amino acid (proline) racemase, after isolation and cloning of a gene from the pathogenic human parasite Trypanosoma cruzi. Although this enzyme is intracellularly located in replicative non-infective forms of T. cruzi, membrane-bound and secreted forms of the enzyme are present upon differentiation of the parasite into non-dividing infective forms. The secreted form of proline racemase is a potent host B-cell mitogen supporting parasite evasion of specific immune responses. Here we describe that the TcPRAC genes in T. cruzi encode functional intracellular or secreted versions of the enzyme exhibiting distinct kinetic properties that may be relevant for their relative catalytic efficiency. Although the $K_m$ values varied between $2 \times 10^{-4}$ and $5.3 \times 10^{-5}$ mol of L-proline/$s$/0.125 $\mu$mol of homodimeric recombinant protein, studies with the enzyme-specific inhibitor and abrogation of enzymatic activity by site-directed mutagenesis of the active site Cys$^{256}$ residue reinforced the potential of proline racemase as a critical target for drug development against Chagas' disease. Finally, we propose a protein signature for proline racemases and suggest that the enzyme is present in several other pathogenic and non-pathogenic bacterial genomes of medical and agricultural interest, yet absent in mammalian host, suggesting that inhibition of proline racemases may have therapeutic potential.

D-Amino acids have long been described in the cell wall of Gram-positive and especially Gram-negative bacteria, where they constitute essential elements of the peptidoglycan and as substitutes of cell wall teichoic acids (1). Moreover, various types of D-amino acids were discovered in a number of small peptides made by a variety of microorganisms through non-ribosomal protein synthesis (2) that function mainly as antibiotic agents. However, these examples were considered exceptions to the rule of homochirality, and a dogma persisted that only L-amino acid enantiomers were present in eukaryotes, apart from a very low level of D-amino acids from spontaneous racemization because of aging (3). Recently, an increasing number of studies have reported the presence of various D-amino acids either as protein-bound (4) or under free forms (5) in a wide variety of organisms, including mammals. The origin of free D-amino acids is less clear than that of protein bound D-amino acids. For instance, in mammals, free D-amino acids may originate from exogenous sources (as described in Ref. 6), but the recent discovery of amino acid racemases in eukaryotes has also uncovered an endogenous production of D-amino acids, questioning their specific functions. Thus, the level of D-aspartate is developmentally regulated in rat embryos (7); the binding of D-serine to N-methyl-D-aspartate mouse brain receptors promotes neuromodulation (8, 9) and D-aspartate appears to be involved in hormonal regulation in endocrine tissues (10). All amino acid racemases require pyridoxal phosphate as a co-factor except proline and hydroxyproline racemases, which are co-factor-independent enzymes. Two reports have been published addressing the biochemical and enzymatic characteristics of the proline racemase from the Gram-positive bacterium Clostridium sticklandii (11, 12). A reaction mechanism was proposed whereby the active site Cys$^{256}$ forms a half-reaction site with the corresponding cysteine of the other monomer in the active, homodimeric enzyme.

Although a variety of racemases and epimerases has been demonstrated in bacteria and fungi, we recently described the first eukaryotic amino acid (proline) racemase isolated from the infective metacyclic forms of the parasitic protozoan Trypanosoma cruzi, the causative agent of Chagas’ disease in humans (13). This parasite-secreted proline racemase (TcPRAC) was shown to be a potent mitogen for host B cells and plays an important role in T. cruzi immune evasion and persistence through polyclonal lymphocyte activation (13). This protein, previously annotated as TcPA45, with monomer size of 45 kDa, is only expressed and released by infective metacyclic forms of the parasite. The genomic organization and transcription of TcPRAC proline racemase gene indicated the presence of two homologous genes per haploid genome (TcPRACA and TcPRACB). Furthermore, localization studies using specific antibodies directed to 45-kDa TcPRAC protein revealed that an intra-
cellular and/or membrane-associated isoform, with monomer size of 39 kDa, is expressed in non-infective epimastigote forms of the parasite. Computer-assisted analysis of the TcPRACA gene sequence suggested that it could give rise to both isoforms (45 and 39 kDa) of parasite proline racemases through a mechanism of alternative trans-splicing, one of which would contain a signal peptide (19). In addition, preliminary analysis of putative TcPRACB gene sequences had revealed several differences that contrast point mutations as compared with TcPRACA but that also suggest that TcPRACB gene could only encode an intracellular isoform of the enzyme as the gene lacks the export signal sequence. Any of these molecular mechanisms per se would ensure the differential expression of intracellular and extracellular isoforms of proline racemases produced in different T. cruzi developmental stages.

Primarily it was essential to elucidate whether TcPRACB gene could encode a functional proline racemase. To answer this question we have expressed TcPRACA and TcPRACB parologue genes in Escherichia coli and performed detailed studies on biochemical and enzymatic characteristics of the recombinant proteins. We show here that TcPRACB indeed encodes a functional proline racemase that exhibits slightly different kinetic parameters and biochemical characteristics when compared with TcPRACA enzyme. Enzymatic activities of the respective recombinant proteins showed that the 39-kDa intracellular isoform of proline racemase produced by TcPRACB construct is more stable and has a higher rate of D/L-proline interconversion than the 45-kDa isoform produced by TcPRACA. Additionally, the dissociation constant of the enzyme-inhibitor complex (K_i) obtained with pyrrole-2-carboxylic acid, the specific inhibitor of proline racemases, is lower for the recombinant TcPRACB enzyme. Moreover, we show that Cys^320 is a key amino acid of the proline racemase active site, because the activity of the enzyme is totally abolished by site-directed mutagenesis of this residue. Finally, multiple alignment of proline racemase amino acid sequences allowed the definition of protein signatures that can be used to identify putative proline racemases in other microorganisms. The significance of the presence of proline racemase in eukaryotes, particularly in T. cruzi, is discussed, as well as the putative consequences of this enzymatic activity in the biology and infectivity of the parasite.

**EXPERIMENTAL PROCEDURES**

**Cloning and Automated Sequencing**—A phage and plasmid DNA were prepared using standard techniques, and direct sequencing was accomplished with the Big dye Terminator kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Extension products were run for 7 h in an ABI 373 automated sequencer. Briefly, to obtain the full length of the TcPRAC gene, 39-labeled 229-bp PCR product was used as a probe to screen a T. cruzi clone CL Brener A Fix II genomic library (see details in Ref. 13). We isolated four independent positive phages. Restriction analysis and Southern blot hybridization showed two types of genomic fragments, each represented by two phages. Complete sequence and flanking regions of representative phages for each pattern were done. Complete characterization of TcPRACA gene, representing the first phage type, was described previously in Ref. 13. Full sequence of the putative TcPRACB gene, representing the second phage type, was then performed, and primers internal to the sequence were used for sequencing as described before (13).

**Chromoblot**—Epimastigote forms of T. cruzi (clone CL Brener) are maintained by weekly passage in LIT medium. Agarose (0.7%) blocks containing 1 x 10^7 cultured parasites were lysed with 0.5 x EDTA/10 mM Tris/1% sarcosyl, pH 8.0, digested by protease K and washed in 10 mM Tris/10 mM EDTA, pH 8.0. Pulsed field gel electrophoresis was carried out at 19°C using the Gene Navigator apparatus (Amersham Biosciences) in 0.5 X TBE. Electrophoresis were performed as described (14). Gels were then stained with ethidium bromide, photographed, exposed to UV light (265 nm) for 5 min, and further blotted under alkaline conditions to a nylon filter (HybondN+; Amersham Biosciences). DNA probe, obtained by PCR amplification of TcPRACA gene with Hi-45 (5’ CTC TCC CAT GGG CCA GGA AAA GCT TCT G 3’) and Bd-45 (5’ GTG ATC ACC AGA TCA/T ACT GC 3’) oligonucleotides (as described in Ref. 13), was labeled with dATP using a Megaprime DNA labeling system (Amersham Biosciences). The chromoblot was hybridized overnight in 2 x Denhardt’s/5% saline/sodium phosphate/EDTA/1.5% SDS at 55°C and washed in 2 x saline/sodium phosphate/EDTA/0.1% SDS followed by 1 x saline/sodium phosphate/EDTA at 60°C. Autoradiography was obtained by overnight exposure of the chromoblot using a PhosphorImager cassette (Molecular Dynamics).

**Plasmid Construction and Protein Purification**—To express TcPRACA gene fragment starting at codon 30 was obtained by PCR, using Hi- and Bd-45 primers, and cloned in frame with a C-terminal His_{6} tag into the pET28b (Molecular Biology Resources, France). The fragment encoding for the TcPRACB consituted of a HindIII digestion of TcPRACB gene fragment obtained by similar PCR and cloned in-frame with a C-terminal His_{6} tag into the pET28b (Molecular Biology Resources, France) following the manufacturer’s instructions.

**Size exclusion Chromatography**—To express TcPRACB proteins were purified as described above and dialyzed against phosphate-buffered saline, pH 7.4, or 0.2 mM NaOAc, pH 6.0, elution buffers in dialysis cassettes (Slide-A-Lyzer T, Pierce), overnight at 4°C. The final protein concentration was adjusted to 2 mg/ml, and 0.5 ml of the solution was loaded onto Amersham Biosciences Superdex 75 column (HR10 x 30), calibrated previously with a medium range protein calibration kit (Amersham Biosciences). Size exclusion chromatography was carried out using a fast protein liquid chromatography system equipped with an Pharmacia System (Amersham Biosciences). Elution was performed at a constant flow rate of 0.5 ml/min, protein fractions of 0.5 ml were collected, and the absorbance was monitored at 280 nm. Each fraction was assayed in racemization assays as described below. Fractions B1 and B5 were reloaded in the Superdex 75 column and submitted to a further size exclusion chromatography to verify the purity of the fractions.

**Racemization Assays**—The percent of racemization with different concentrations of L-proline, t-proline, L-hydroxy (OH)-proline, D-hydroxy (OH)-proline was calculated as described (13) by incubating a 500-μl mixture of 0.25 μM dimeric protein and 40 mM substrate in 0.2 M sodium acetate, pH 6.0, for 30 min or 1 h at 37°C. The reaction was stopped by incubating for 10 min at 80°C and freezing. Water (1 ml) was then added, and the optical rotation was measured in a polarimeter 241MC (PerkinElmer Life Sciences) at a wavelength of 365 nm, in a cell with a path length of 10 cm, at a precision of 0.001°. The percent of racemization of 40 mM t-proline as a function of pH was determined using 0.2 M sodium acetate, potassium phosphate, and Tris-HCl buffers; reactions were incubated 30 min at 37°C as described above. All reagents were purchased from Sigma.

**Kinetic Assays**—Concentrations of L- and t-proline were determined polarimetrically from the optical rotation of the solution at 365 nm in a cell of 10-cm path length, thermostated at 37°C. Preliminary assays were done with 40 mM L-proline in 0.2 M sodium acetate, pH 6.0, in a final volume of 1.5 ml. Optical rotation was measured every 5 s during 10 min and every 5 min to 1 h. After determination of the linear part of the curve, velocity in 5–160 mM substrate was measured every 30 s during 10 min to determine K_{m} and V_{max}. Calculations were done using the Kaleidagraph program. Inhibition assays were done by incubating each dimeric protein, 6.7 μg/ml pyrrole-2-carboxylic acid (PAC), (15) to 20 to 160 mM t-proline, as described above. Graphic representation and linear curve regression allowed the determination of K_{i} as [PAC]/[slope with PAC]/[slope without PAC] – 1. All reagents were purchased from Sigma.

**Site-directed Mutagenesis of C. sticklandii TcPRACA**—Site-directed mutagenesis was performed by PCR, adapting the method of Higuchi et al. (15). Briefly, mutation of Cys^320 of the proline racemase active site was produced by two successive polymerase chain reactions based on site-directed mutagenesis using two overlapping mutagenic primers, act-1 (5’ GCC GAT CGC TCT AGC AAG GCA GCC ACC 3’) and act-2 (5’ GGT GCC TGG CCA GCC CAG GAG 3’), designed to introduce a single codon mutation in the active site cysteine (TGT) at position 320 by a serine (AGC). A first step

1The abbreviations used are: PAC, pyrrole-2-carboxylic acid; CsPR, C. sticklandii proline racemase.
FIG. 1. Comparative analysis of sequences of *T. cruzi* TcPRACA and TcPRACB proline racemase isoforms. A, alignment of TcPRACA (Tc-A) and TcPRACB (Tc-B) nucleotide sequences. Non-coding sequences are shown in italics; trans-splicing signals are underlined; putative spliced leader acceptor sites are double underlined; the region encoding the computer-predicted signal peptide is indicated by a double-headed Trypanosoma cruzi Proline Racemases
standard PCR amplification was performed using the TcPRACA DNA as template and a mixture of act-1 primer and the reverse C terminus primer, Bg-45 (5'-CTG AGC TCG ACC AGA TCA A ACT GC 3') (codon 423) or a mixture of act-2 primer and the forward N terminus primer, Hi-45 (5'-CTC TCC CAT GGG GCA GGA AAA GCT TCT G 3') (codon 53) (see Fig. 5). Resulting amplified fragments of 316 and 918 bp, respectively, were purified by a Qiagen PCR extraction kit as prescribed by the manufacturer. 

RESULTS
Expression of a Functional Intracellular Isoform of Proline Racemase—We have previously characterized (13) a TcPRAC gene from T. cruzi and demonstrated in vivo and in vitro that it encodes a proline racemase enzyme. Analysis of the genomic organization and transcription of the TcPRAC gene indicated the presence of two paralogue gene copies per haploid genome, named TcPRAC (GenBank™ accession number AF195522) and TcPRACB (GenBank™ accession number AY140947). We showed that TcPRAC encodes a functional co-factor-independent proline racemase, closely resembling the C. sticklandii proline racemase (CsPR) (GenBank™ accession number E101199) (11). We now sequenced the full-length of TcPRAC and, as can be observed in Fig. 1A, TcPRAC and TcPRACB genes both possess the characteristic trypanosome polypyrrolidine-rich motifs in the intergenic region that are crucial trans-splicing signals when located upstream of an (AG)-dinucleotide used as acceptor site. As in other T. cruzi genes, UUA triplets are found at the end of the 3' untranslated region preceding the initiation codon. Amino acid changes are indicated above and below the vertical lines, and their positions in the sequence are shown in parentheses. SP, signal peptide; \N terminal and C terminal are indicated for both proteins. C, hydrophobicity profile of TcPRAC. Dotted line depicts the cleavage site as predicted by Von Heijne’s method (amino acids 31–32). D, ethidium bromide-stained gel of chromosomal bands of T. cruzi CL Brener clone after separation by pulsed-field gel electrophoresis (lane 1) and Southern blot hybridization with TcPRAC probe (lane 2). The sizes (Mb) of chromosomal bands are indicated, as are the region chromosome numbers (in roman numerals).
To produce C-terminal His 6-tagged recombinant proline racemase, we expressed both Cano VII and V, respectively, according to the numbering system of migrating bands of other protein-coding genes in sequence. Furthermore, while differing by only seven amino acids, whose translation would start at the ATG codon at position 274, the intracellular isoform of the protein is indeed functional in vivo, because proline racemase enzymatic activity was displayed, and levels of racemization were dependent on protein concentration.

| Protein       | Column | % of preservation of proline racemase activity | NaOAc, pH 6 | Table I: Stability of recombinant TcPRACA and TcPRACB proline racemases under different storage conditions |
|---------------|--------|-----------------------------------------------|-------------|----------------------------------------------------------------|
|               | CTRL   | RT                             | +4 °C       | Gly+20 °C | 4 °C   | (NH₄)₂SO₄ |
| rTcPRACA      | 100.0  | 16.0                           | 66.5        | 62.9   | 31.0   | 53.9       | 100.0 |
| rTcPRACB      | 100.0  | 100.0                          | 34.0        | 93.6   | 77.6   | 98.4       | 100.0 |

After purification on nickel-nitrioltriacetic acid-agarose column, recombinant proteins were kept for 10 days in nickel column buffer (20 mM Tris/500 mM NaCl/500 mM imidazol, pH 8.0) at room temperature (RT) or at +4 °C or diluted either in 50% glycerol and maintained at −20 °C (Gly−20 °C) or in optimum pH buffer (NaOAc, pH 6.0) at 4 °C. Recombinant enzymes were precipitated in (NH₄)₂SO₄ and kept in solution at 4 °C or pellet dried at −20 °C. Racemase assays were performed for 30 min at 37 °C. Percent of preservation was determined polarimetrically using 0.25 μl of either purified rTcPRACA or rTcPRACB enzymes and 40 μl of L-proline, as compared with results obtained with freshly purified proteins (CTRL). This results are representative of at least two independent experiments.
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The conservation of critical proline racemase protein signatures and putative proline racemase isoforms. The progress of racemization reaction was monitored polarimetrically, as described previously (13). A, the determination of the linear part of the curve was performed at 37 °C in medium containing 0.2 mM sodium acetate, pH 6.0, 0.25 mM purified enzyme, and 40 mM L-proline. rTcPRACA reactions are represented by black squares, and rTcPRACB reactions are represented by white squares. B, initial rate of racemase activity was assayed at 37 °C in medium containing 0.2 mM sodium acetate, pH 6.0, 0.125 mM rTcPRACA (solid squares), or rTcPRACB (open squares) purified enzymes and different concentrations of L-proline. Lineweaver-Burk double reciprocal plots were used to determine values for $K_m$ and $V_{max}$ where $1/V$ is plotted in function of $1/[S]$, and the slope of the curve represents $K_m/V_{max}$. Values obtained were confirmed by using the Kaleidagraph® program and Michaelis-Menten equation. The values are representative of six experiments with different enzyme preparations. C, double reciprocal plot kinetics of 0.125 mM rTcPRACA proline racemase isoform in the presence (open) or absence (closed) of 6.7 μM PAC competitive inhibitor in function of L-proline concentration. For comparison, $K_m$ reported for the proline racemase of C. sticklandii was 2.3 mM; kinetic assays using the native protein obtained from a soluble epimastigote fraction revealed a $K_m$ of 10.7 mM and a $K_0$ of 1.15 μM.

Requirement of a Dimeric Structure for Proline Racemase Activity—When rTcPRACA was submitted to size exclusion chromatography on a Superdex 75 column at pH 6.0, two peaks of protein were eluted around 80 kDa (B2 fraction) and 43 kDa (B4 fraction), respectively, presumably corresponding to dimeric and monomeric forms of the enzyme (Fig. 4). Western blot analysis of whole T. cruzi epimastigote extracts using non-denaturing PAGE had previously indicated a molecular mass of 80 kDa for the native protein (not shown), whereas a 45-kDa band was obtained by SDS-PAGE (13). To eliminate cross-contamination, B1 and B5 fractions, eluted at the start and at the end of the predicted dimer (B2) or monomer (B4) peaks, respectively, were reloaded onto the column, and the profiles obtained (see Fig. 4, insets) confirmed the purity of the fractions. Enzyme activity resides in the 80-kDa peak but not in the 43-kDa peak (Table II). These results corroborated that two subunits of the protein are necessary for racemase activity. At neutral pH (7.4 or above), the rTcPRACA gives rise to high molecular weight aggregates that are not observed with rTcPRACB, consistently with its broader optima pH spectrum (not shown).

Abrogation of Proline Racemase Activity by Mutation of Cys$^{330}$ of the Catalytic Site—C. sticklandii proline racemase is described as a homodimeric enzyme with subunits of 38 kDa and a single proline binding site for every two subunits, where two cysteines at position 256 might play a crucial role in catalysis by the transfer of protons from and to the bound substrate (12). We have shown previously (16) that mitogenic properties of the T. cruzi proline racemase are dependent on the integrity of the enzyme active site, as inhibition of B-cell proliferation is obtained by substrate competition and specific use of analogues (PAC) resembling the structure assumed by the substrate proline in its transition state. To verify the potential role of the cysteine residues at the active site of the T. cruzi proline racemase, we replaced Cys$^{330}$ by a serine residue through site-specific mutation of TcPRACA. The choice of serine as the substituting amino acid was made to avoid further major disturbances on three-dimensional structure of the protein (see strategy in Fig. 5 and “Experimental Procedures”). After confirmation of the single codon mutation through sequencing of the construct (not shown), the C$^{130S}$TcPRACA mutant proline racemase was expressed in E. coli and purified in the same manner as wild-type rTcPRACA. We then used C$^{130S}$TcPRACA in racemization assays to verify the effects of the mutation on the enzymatic activity of the protein. As can be observed in Table III, a total loss of proline racemase activity is observed as compared with the wild-type enzyme, establishing that proton transfer during proline racemization is specifically dependent on the presence of the cysteine residue in the active site.

Proline Racemase Protein Signatures and Putative Proline Racemase Sequences in Databases—The conservation of critical
residues between parasite and bacterial proline racemases prompted us to search for similarities between \( Tc \) PRAC and other protein sequences in SWISS-PROT and TrEMBL databases. Twenty-one protein sequences yielded significant homologies, from 11 organisms, such as several proteobacteria of the \( \gamma \)-subdivision (\( \text{Agrobacterium} \), \( \text{Brucella} \), \( \text{Rhizobium} \)) and \( \gamma \)-subdivision (\( \text{Xanthomonas} \) and \( \text{Pseudomonas} \)), as well as of the \( \beta \)-subdivision (\( \text{Streptomyces} \) and \( \text{Clostridium} \)). Within the eukaryota, besides in \( T. \) cruzi, homologous genes were detected in the human and mouse genomes, where predicted proteins show overall similarities with proline racemase. Except for \( C. \) sticklandii and \( X. \) campestris, each other organism encodes two paralogs, and \( A. \) tumefaciens contains three genes. The multiple alignment also allowed for the definition of three signatures of proline racemase, which are described here in PROSITE format. As can be seen in Table IV, when using a minimal motif of proline racemase protein (M I), \([IVL][GD]XHXXG[ENM][XX][RD][X][VI][XX]G\), located immediately after the start codon at position 79, we obtained nine hits. A second motif (M II), consisting of \([NSM][VA][EP][AS][FY]X(13, 14)[GK]X[IVL][X][IV][AS][YFW][GGX][FWY]\), starting at position 218, gave 14 hits; however, the first or the second half of this motif is not sufficiently stringent to be restrictive for putative proline racemases but gives hits for different protein families. A third motif (M III), from positions 326 to 339, namely \( \text{DRSPXG[GAG]XXX} \), was considered as a minimal pattern. Note that in position 330, the cysteine of the active site was replaced by an \( X \). As shown in Table IV, this minimal
pattern yields all 21 hits. Curiously, both genes in human, as well as in mouse, encode threonine instead of cysteine at the X position in motif III, whereas in Brucella, Rhizobium, and Agrobacterium species, each encode one protein with C and one with T in this position. We cannot hypothesize the implications of this substitution for the functionality of these putative proteins. If the residue at position 330 is maintained as a cysteine in motif I, II, and III, a reduced number of 12 hits from nine organisms is thus obtained, which can probably be considered as true proline racemases. The alignment of the 21 protein sequences and derived cladogram are shown in Fig. 6 and Fig. 7, respectively, and the three boxes depicted correspond to motifs I, II, and III described above. We thus propose DRSPCGXGXXXXX, Agrobacterium tumefaciens, and Xanthomonas campestris, as the minimal signature for proline racemases. BLAST searches against unfinished genomes yielded, at present, an additional 13 predicted protein sequences from 9 organisms, with high similarity to proline racemases, all containing motif III. Organisms are Clostridium difficile, Clostridium botulinum, Bacillus anthracis, Brucella suis, Pseudomonas putida, Rhodobacter sphaeroides, Burkholderia pseudomallei, Burkholderia mallei, and the fungus Aspergillus fumigatus. These results indicate that proline racemases might be quite widespread.

DISCUSSION

Proline racemase, an enzyme previously only described in protobacterium C. sticklandii (11), was shown to be encoded also by the eukaryote T. cruzi, a highly pathogenic protozoan parasite (13). The TcPRAC (T. cruzi proline racemase), formerly called TcPA45, is an efficient mitogen for host B cells and is secreted by the metacyclic forms of the parasite upon infection, contributing to its immune evasion and persistence through nonspecific polyclonal lymphocyte activation (13). Our previous results (13) suggested that TcPRAC is encoded by two paralogous genes per haploid genome. Protein localization studies have also indicated that T. cruzi can differentially express intracellular and secreted versions of TcPRAC during cell cycle and differentiation, as the protein is found in the cytoplasm of non-infective replicative (epimastigote) forms of the parasite, and bound to the membrane or secreted in the infective, non-replicative (metacyclic trypomastigote) parasites (13). Here we have characterized the two TcPRAC paralogs and demonstrated that both TcPRACA and TcPRACB give rise to functional isoforms of co-factor-independent proline racemases, which display different biochemical properties that may well have important implications in the efficiency of the respective enzymatic activities. As suggested previously (11, 17, 18) by biochemical and theoretical studies for the bacterial proline racemase, our studies reveal that TcPRAC activities rely on two monomeric enzyme subunits that perform interconversion of L- and/or D-proline enantiomers by a two-base mechanism reaction in which the enzyme removes an α-hydrogen from the substrate and donates a proton to the opposite side of the α-carbon. It has been predicted that each subunit of the homodimer furnishes one of the sulfhydryl groups (18). In the present work we showed that TcPRAC enzymatic activities are bona fide dependent on the Cys330 residue of the active site, as site-specific C330S mutation totally abrogates L- and D-proline racemization, in agreement with our previous demonstration

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| Organism                  | Seq | Access. nb | M I | M II | M III | M III* |
|---------------------------|-----|------------|-----|------|-------|-------|
| Agrobacterium tumefaciens | 1   | Q8UIA0     | +   | +    | +     | +     |
| Agrobacterium tumefaciens | 2   | Q8UIX2     | -   | -    | -     | -     |
| Agrobacterium tumefaciens | 3   | Q8U93Y5    | -   | +    | +     | +     |
| Brucella melitensis       | 1   | Q8YF29     | -   | +    | +     | +     |
| Brucella melitensis       | 2   | Q8YF6D     | -   | +    | +     | +     |
| Clostridium sticklandii   |     | Q6L4Q3     | -   | +    | +     | +     |
| Homo sapiens              | 1   | Q86EM0     | +   | +    | -     | -     |
| Homo sapiens              | 2   | Q86LJ5     | -   | +    | +     | +     |
| Mus musculus              | 1   | Q8CXA2     | +   | +    | +     | +     |
| Mus musculus              | 2   | Q89KB5     | +   | +    | +     | +     |
| Pseudomonas aeruginosa    | 1   | Q94J76     | +   | +    | +     | +     |
| Pseudomonas aeruginosa    | 2   | Q94I89     | -   | -    | +     | +     |
| Rhizobium loti            | 1   | Q9SF29     | +   | +    | +     | +     |
| Rhizobium loti            | 2   | Q9SF5B     | +   | +    | +     | +     |
| Rhizobium meliloti        | 1   | Q82WR9     | -   | -    | +     | +     |
| Rhizobium meliloti        | 2   | Q82WS1     | -   | +    | +     | +     |
| Streptomyces coelicolor   |     | Q83RX9     | +   | +    | -     | -     |
| Trypanosoma cruzi         | 1   | Q8NF5C     | +   | +    | +     | +     |
| Trypanosoma cruzi         | 2   | a           | +   | +    | +     | +     |
| Xanthomonas axonopodis    | 1   | Q8PJ1      | -   | -    | +     | +     |
| Xanthomonas axonopodis    | 2   | Q8FKE4     | -   | -    | +     | +     |
| Xanthomonas campestris    |     | Q8F933     | -   | -    | +     | +     |

* GenBank™ accession number AY140947.
that TcPRAC enzymatic activity is abolished through alkylation with iodoacetate or iodoacetamine, similarly to the Clostridium proline racemase, where carboxymethylation was shown to occur specifically with the two cysteines of the reactive site leading to enzyme inactivation (12). Although gene sequence analysis predicted that by a mechanism of alternative splicing TcPRACA could generate both intracellular and secreted versions of parasite proline racemase, our present stud-

FIG. 6. Sequence alignments of proteins (Clustal X) obtained by screening SWISS-PROT and TrEMBL databases using motifs I, II, and III. Amino acids involved in M I, M II, and M III are shaded in dark gray. The 13–14 unspecific amino acids involved in M II are shaded in light gray. SWISS-PROT accession numbers of the sequences are in Table IV.
ies demonstrate that TcPRACB gene sequence per se codes for a protein lacking the amino acids involved in peptide signal formation and an extra N-terminal domain present in TcPRACA protein, resembling more closely the CePR. Thus, TcPRACB can only generate an intracellular version of TcPRAC proline racemase.

Interestingly, the presence of two homologous copies of TcPRAC genes in the T. cruzi genome, coding for two similar but with distinct specific biochemical properties, could reflect an evolutionary mechanism of gene duplication and a parasite strategy to ensure a better environmental flexibility. This assumption is comforted by the potential of TcPRACA gene to generate two related protein isoforms by alternative splicing, a mechanism that is particularly adept for cells that must respond rapidly to environmental stimuli. Primarily, trans-splicing appears indeed to be an ancient process that may constitute a selective advantage for split genes in higher organisms (19), and alternative trans-splicing was only proven to occur in T. cruzi recently (20). As an alternative for promoter selection, the regulated production of intracellular and/or secreted isoforms of proline racemase in T. cruzi by alternative trans-splicing of TcPRACA gene would allow the stringent conservation of a constant protein domain and/or the possibility of acquisition of an additional secretory region domain. As a matter of fact, our recent investigations using RT-PCR based strategy and a common 3′ probe to TcPRACA and TcPRACB sequences combined to a 5′ spliced leader oligonucleotide followed by cloning and sequencing of the resulting fragments have indeed proved that an intracellular version of TcPRAC may also originate from the TcPRACA gene, corroborating this hypothesis.2,3

Gene duplication is a relatively common event in T. cruzi that adds complexity to parasite genomic studies. Moreover, TcPRAC chromosomal mapping revealed two chromosomal bands that possess more than three chromosomes each and that may indicate that proline racemase genes are mapped in size-polymorphic homologous chromosomes, an important finding for proline racemase gene family characterization. Preliminary results in this laboratory have, for instance, revealed that T. cruzi DM28c type I strain maps proline racemase genes to the same chromoblot regions identified with T. cruzi CL type II strain used in the present work. Other isolates of the parasites are presently under investigation (data not shown).

It is well known that proline constitutes an important source of energy for several organisms, such as several hemoflagellates (21–23), and for flight muscles in insects (24). Furthermore, a proline oxidase system was suggested in trypanosomes (25), and the studies reporting the abundance of proline in triatominiae guts (26) have implicated proline in metabolic pathways of T. cruzi parasites, as well as in its differentiation in the digestive tract of the insect vector (27). Thus, it is well accepted that T. cruzi can use L-proline as a principal source of carbon (25). Moreover, our preliminary results using parasites cultured in defined media indicate that both epimastigotes, found in the vector, and infective metacyclic trypomastigote forms can efficiently metabolize L- or D-proline as the sole source of carbon (not shown). Although certain reports indicate that biosynthesis of proline occurs in trypanosomes, i.e. via reduction of glutamate carbon chains or transamination reactions, our results reveal that an additional and direct physiological regulation of proline might exist in the parasite to control amino acid oxidation and its subsequent degradation or yet to allow proline utilization. In fact, a recent report (28) showed two active proline transporter systems in T. cruzi. We suggest that T. cruzi proline racemase may possibly play a consequential role in the regulation of intracellular proline metabolic pathways, or else it could participate in mechanisms of post-translational addition of D-amino acid to polypeptide chains. On one hand, these hypotheses would allow for an energy gain and, on the other hand, would permit the parasite to evade host responses. In this respect, it was reported that a single D-amino acid addition in the N terminus of a protein is sufficient to confer general resistance to lytic reactions involving host proteolytic enzymes (29). The expression of proteins containing D-amino acids in the parasite membrane would benefit the parasite inside host cell lysosomes, in addition to the contribution to the initiation of polyclonal activation, as described previously (30, 31) for polymers composed of D-enantiomers. Although D-amino acid inclusion in T. cruzi proteins would benefit the parasite, this hypothesis remains to be proven, and direct evidences are technically difficult to obtain.

It is worth noting that metacyclogenesis of epimastigotes into infective metacyclic forms involves parasite morphologic changes that include the migration of the kinetoplast, a structure that is physically linked to the parasite flagellum, and many other significant metabolic alterations that combine to confer infectivity/virulence to the parasite (13, 32). Proline racemase was shown to be preferentially localized in the flagellar pocket of infective parasite forms after metacyclogenesis (13), as are many other known proteins secreted and involved in early infection (33). It is also conceivable that parasite proline racemase may function as an early mediator for T. cruzi differentiation through intracellular modification of internalized environmental free proline, as suggested above and already observed in some bacterial systems. As an illustration, exogenous alanine has been described as playing an important role in bacterial transcriptional regulation by controlling an operon formed by genes coding for alanine racemase and a smaller subunit of bacterial dehydrogenase (34). In bacteria, membrane alanine receptors are responsible for alanine and proline entry into the bacterial cell (35). We can then hypothesize that the availability of proline in the insect gut milieu associated to a mechanism of environmental sensing by specific receptors in the parasite membrane would stand for parasite proline uptake and its further intracellular racemization. Proline racemase would then play a fundamental role in the reg-

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2 N. Chamond, N. Coatnoan, J. C. Barale, A. Cosson, A. Berneman, W. Degrave, and P. Minogrio, manuscript in preparation.

3 The proline racemase/B-cell mitogen of T. cruzi is a virulence factor whose mRNA is regulated differentially through development by alternative splicing.
ulation of parasite growth and differentiation by its participation in both metabolic and toxic pathways and the expression of proteins containing D-proline, as described above, consequently conferring parasite infectivity and its ability to escape host-specific responses.

Thus far, and contrasting to the intracellular isoform of TcPRAC found in epimastigote forms of T. cruzi, the ability of metacyclic and bloodstream forms of the parasite to express and secrete proline racemase may have further implications in host-parasite interaction. In fact, the parasite-secreted isoform of proline racemase participates actively in the induction of nonspecific polyolonal B-cell responses upon host infection (13) and favors parasite evasion, thus ensuring its persistence in the host. As described for other mitogens and parasite antigens (36–38), and in addition to its mitogenic property, TcPRAC could also be involved in modifications of host cell targets enabling better parasite attachment to host cell membranes, in turn assuring improved infectivity. Because several reports associate accumulation of L-proline with muscular dysfunction (39) and inhibition of muscle contraction (40), the release of proline racemase by intracellular parasites could alternatively contribute to the maintenance of infection through regulation of L-proline concentration inside host cells, as proline was decribed as essential for the integrity of muscular cell targets.

Therefore, we have demonstrated recently that transgenic parasites hyperexpressing TcPRACA or TcPRACB genes, but not functional knock-outs, are five to ten times more infective to used less stringent signatures for proline racemase is striking. Likewise, previous functional knock-outs, are five to ten times more infective to T. cruzi Proline Racemases

alanine racemase and D-amino acid oxidase Lysteria reports (41) demonstrated that genetic inactivation of proteins containingD-proline, as described above, consequently associate accumulation ofL-proline with muscular dysfunction (36

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T. cruzi –

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Biochemical Characterization of Proline Racemases from the Human Protozoan Parasite *Trypanosoma cruzi* and Definition of Putative Protein Signatures
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*J. Biol. Chem.* 2003, 278:15484-15494.
doi: 10.1074/jbc.M210830200

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