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Cloning, heterologous expression, and substrate specificities of protein farnesyltransferases from *Trypanosoma cruzi* and *Leishmania major*☆

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Abstract

Chagas disease and leishmaniasis are tropical diseases caused by the protozoan parasites, *Trypanosoma cruzi* and *Leishmania* species, respectively. Protein farnesyltransferase (PFT) is being investigated as a target for anti-trypanosomatid agents because inhibitors of this enzyme are highly toxic to these parasites compared to mammalian cells. Here, we report the cloning of the α- and β-subunit genes of PFT from *T. cruzi* and *Leishmania major*. The proteins encoded by these genes are considerably larger than those of mammalian PFTs due to the presence of a number of inserts of >25 amino acids that map to junctions between helical structural elements. These inserts are not part of the active site or the interface between the two subunits. Northern blots demonstrate expression of messenger RNA for the PFT subunits in both mammalian and insect life-cycle stages of these parasites. The *T. cruzi*, *Trypanosoma brucei*, and *L. major* PFTs were overexpressed in the Sf9 cell/baculovirus system as active enzyme forms. Kinetic studies with a panel of CALX-containing peptides with all 20 amino acids in the X-position show that trypanosomatid PFTs have similar substrate specificities and these are different from the mammalian PFT substrate specificity patterns.

Keywords: Protein prenylation; Farnesylation; Anti-parasitic agents; *Trypanosoma cruzi*; *Trypanosoma brucei*; *Leishmania major*

1. Introduction

Protein prenylation, the attachment of 15-carbon (farnesyl) and 20-carbon (geranylgeranyl) groups to the C-termini of proteins, occurs in mammalians, yeast, plants, and protozoa [1–4], including trypanosomatids. Protein farnesylation involves protein farnesyltransferase (PFT)-catalyzed attachment of the farnesyl group from farnesyl pyrophosphate (FPP) to the cysteine SH of the C-terminal sequence motif CaaX (where C is cysteine, a is usually but not always an aliphatic residue, and X is a variety of different amino acids) [5,6].

Trypanosomatid parasites, including *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* species, are important human pathogens for which adequate chemotherapies are not available. Protein farnesylation is a potentially good drug target for trypanosomatids for the following reasons: (1) protein farnesylation inhibitors have potent activity against cultured forms of these

Abbreviations: FPP, farnesyl pyrophosphate; PFT, protein farnesyltransferase; PGGT-I, protein geranylgeranyltransferase type I; *Lmj*, *Leishmania major*; *La*, *Leishmania amazonensis*.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers AF461504, AF461505, AF461506, AF461507, AF461508.

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parasites and these inhibitors are more toxic against parasite cells than mammalian cells [7,8], (2) for the one trypanosomatid PFT that has been studied, *T. brucei* PFT (*Tb*PFT), the substrate specificities and inhibitor selectivity are distinct from mammalian PFT [7], and (3) intensive effort in the pharmaceutical industry to develop small molecule inhibitors of mammalian PFTs for anti-cancer purposes creates an abundance of compounds that, through collaborations, may be screened for selective activity against parasites [9,10].

The *Tb*PFT was previously purified from insect stage parasites (procyclics) and the *TbPFT* α- and β-subunit genes have been cloned [8,11]. In the current study, the *T. cruzi PFT* (*TcPFT*) and *Leishmania major PFT* (*LmjPFT*) genes were cloned and expressed in a heterologous system. The substrate specificities of the parasite enzymes are compared with the specificity of the rat PFT.

2. Materials and methods

2.1. Cells and culture techniques

T. cruzi Tulahuen strain (gift of Steve Reed, Infectious Diseases Research Institute, Seattle, WA), *L. major* Friedlin strain, *Leishmania amazonensis* LV78 strain (gifts of K.P. Chang, Chicago Medical School), and *L. major* JiSH 118 strain (gift of Simon Croft, London School of Hygiene and Tropical Medicine, London, UK) were used as described below. *T. cruzi* epimastigotes and mammalian stages of *T. cruzi* were grown as described elsewhere [12]. *Leishmania* promastigotes were grown in Medium 199 (Gibco BRL) with 10% heat inactivated fetal calf serum at 27° C. Mammalian stage *L. major* JiSH 118 strain amastigotes were obtained from subcutaneous lesions of BALB/c mice 3 weeks following infection with 2×10^7 stationary phase promastigotes.

2.2. Molecular cloning of PFT subunit genes

2.2.1. TcPFT α- and β-subunit genes

Multiple protein sequence alignments were made with PFT-α homologues from *H. sapiens* (GenBank #1346694), *S. cerevisiae* (GenBank #266880), *A. thaliana* (GenBank #15231672), and *T. brucei* (GenBank #8163922) using Block Maker (http://www.blocks.fhcrc.org/blockmkr/make_blocks.html). Regions corresponding to residues 195–204 and 342–352 of the translated *T. brucei* amino acid sequence were used to design sense (5′-ccgaagaacttccaggtgtggcaycaymg-3′) and anti-sense (5′-acgtagaagcggtggcaccangcygarttrtt-3′) degenerate oligonucleotides using the program CODEHOP (http://www.blocks.fhcrc.org/blocks/codehop.html). An internal portion of the *T. cruzi* gene was amplified by PCR using genomic DNA from the Tulahuen strain [13]. The remaining portions of the gene were amplified from cDNAs by spliced-leader RACE and oligo-dT RACE [14] and sequenced. The full-length gene was then amplified from genomic DNA using high fidelity DNA polymerase, Pwo (Roche Molecular Biochemicals), and sequenced on both DNA strands from two independently amplified clones. A similar approach was taken to clone the *TcPFT* β-subunit gene based on degenerate oligonucleotides designed to amino acid residues 318–329 (sense primer: 5′-gcgtcggaggcccacggcgcgtaccancartgygg-3′) and 485–495 (anti-sense primer: 5′-gacaggccggacagggagtagcangtrtgrta-3′) of the translated *TbPFT* β-subunit gene (GenBank #8163924).

2.2.2. LmjPFT α-subunit gene

Similarly to above, degenerate PCR was performed to amplify an internal portion of the *LmjPFT* α-subunit gene based on *T. brucei* sequence. A ^{32}P-labeled nucleotide probe was made using the PCR fragment, and this probe was used to screen a *L. major* cosmid library provided by the *Leishmania* Genome Network (http://www.ebi.ac.uk/parasites/leish.html) [15]. Cosmid clone L8542 (associated with Chromosome 29) was strongly positive, and this clone was shotgun sequenced (# AC087068) [16]. The full-length gene was amplified from genomic *L. major* DNA and found to have identical sequence to the cosmid clone.

2.2.3. LmjPFT β-subunit gene

Four DNA sequences from *L. major* with high homology to the 3′-end of *TbPFT* β-subunit were detected by BLAST search of GenBank: AQ852316, AZ047899, AL160493, and AL160794. A ^{32}P-labeled probe was made to part of this sequence and hybridized to the *L. major* genomic cosmid library as above. Positive cosmids were identified and mapped to chromosome 26. Cosmids L2522 and L3349 were subjected to DNA sequencing using procedures recommended by the *Leishmania* Genome Network [16]. An open reading frame of 2178 nts was identified, and the gene was amplified from genomic *L. major* Friedlin DNA.

2.3. Northern blots

The sources of RNA were (1) midlog-phase *T. cruzi* epimastigotes, (2) mammalian stages of *T. cruzi* grown in co-culture with murine 3T3 fibroblasts, (3) murine 3T3 fibroblasts, (4) midlog-phase *L. major* JiSH 118 promastigotes, (5) *L. major* JiSH 118 amastigotes obtained directly from mice tissues, and (6) skin and subcutaneous tissue from uninfected region of mice. The *L. major* JiSH 118 strain was employed because of its ability to produce high numbers of tissue amastigotes in mice for a source of RNA. Total RNA was extracted using the RNeasy kit (Qiagen). Approximately 10 μg of

RNA was loaded per lane, electrophoresed on a 1% formaldehyde agarose gel, then blotted to Hybond^{TM}-N nylon membrane (Amersham Life Science). ^{32}P-labeled probes were prepared by random prime labeling of full-length PCR fragments of the respective *PFT* subunits. Blots were washed under high stringency conditions at 42 °C [17]. Mammalian cells (3T3 fibroblasts and murine skin/subcutaneous tissue) did not contain RNA that hybridized with the parasite PFT probes.

2.4. Production of recombinant Tc PFT, Tb PFT, and Lmj PFT in the Sf9/baculovirus system

PFT α- and β-subunit genes from each species were transferred from the pGEM-T cloning vector (Promega) to pFastBac (Gibco BRL). (Allele 1 of the *TcPFT* α-subunit was used in these experiments). The cloning of the *TbPFT* genes was described previously [11]. The subsequent transposition into DH10Bac competent *E. coli*, bacmid purification, sequence validation, and transfection of Sf9 cells were carried out according to manufacturer's instructions for the Bac-to-Bac Baculovirus Expression System (Gibco BRL). Baculoviruses were plaque purified followed by three amplifications to final titers of 10^9–10^{10} pfu ml^{-1}. Sf9 cells were infected with baculoviruses carrying *PFT* α- and β-subunit genes at multiplicities of infection of ~3 for each virus. After incubation for 48–72 h, the cells were collected by centrifugation and frozen at −80 °C. The cells were thawed on ice in lysis buffer (20 mM Tris-HCl, 50 mM NaCl, 5 μM $ZnCl_2$, pH 8.0) containing freshly added 10 mM DTT and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 30 μM each of tosyllysine chloromethyl ketone and tosylphenylalanine chloromethyl ketone, and 10 μg ml^{-1} each of aprotinin, leupeptin, and pepstatin A) and disrupted with a Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min, followed by 10 000 × *g* for 10 min, followed by 120 000 × *g* for 80 min. PFT assays (below) were carried out with the supernatants (0.2 μg protein/reaction).

2.5. Partial purification of native Tc PFT and La PFT

Native *Tc*PFT was partially purified from *T. cruzi* Tulahuen epimastigote cells (5×10^9 cells from a 1-l culture) as described previously [18]. Briefly, the cells were disrupted at 0 °C in 1 mM Tris-HCl, I mM EDTA, 1 mM DTT, pH 8.0, containing protease inhibitors (as above) by sonication with a Branson sonicator. The lysate was supplemented with 20 mM Tris-HCl (pH 8.0), 5 mM DTT, and 5 μM $ZnCl_2$, and the mixture was centrifuged at 120 000 × *g* for 80 min at 4 °C. The supernatant was subjected to protein precipitation with 60% saturated ammonium sulfate at 0 °C. After dialysis against 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, the 0–60% ammonium sulfate fraction was loaded onto a column (1 × 8 cm) of Q-Sepharose FF. The protein was eluted with a NaCl gradient from 50 to 600 mM in the same buffer. The fractions containing PFT activity were pooled and concentrated in a Centricon 30 (Amicon). The concentrated sample was frozen in liquid nitrogen and stored at −80 °C. Typical yield of *Tc*PFT activity was 1.7 microunits.

Similarly, native *La*PFT was partially purified from *L. amazonensis* promastigote cells (~5×10^9 cells from a 1-l culture). *La*PFT was eluted as a single peak at ~0.4 M NaCl during Q-Sepharose chromatography. Typical yield of *La*PFT is 0.7 microunits per 5×10^9 cells. Native *Tb*PFT was purified from procyclic form cells of *T. brucei brucei* EATRO140 as described previously [7].

2.6. PFT Assay

The standard reaction mixture contains 5 μM RAS1-CVIM [8], 0.75 μM (0.3 μCi) [^{3}H]FPP (20 Ci mmol^{-1}, American Radiolabeled Chemicals) and an appropriate amount of PFT in 20 μl of buffer (30 mM potassium phosphate, 5 mM DTT, 0.5 mM $MgCl_2$, 20 μM $ZnCl_2$, pH 7.7). The mixture was incubated at 30 °C for 15 min, and the amount of the radioactive product was quantified by the glass-fiber filter method [19]. One microunit of PFT is defined as the amount of the enzyme that produces 1 pmol of the product per min under the standard conditions.

2.7. Substrate specificity studies with SSCALX peptides

Twenty different peptides SSCALX where X is one of 20 naturally occurring amino acids were prepared by Synpep (Dublin, CA) and United Biochemicals Research (Seattle, WA), and purified by C18 reverse phase HPLC as described previously [11]. Each of the 20 different SSCALX peptides (10 μM) was incubated at 30 °C for 30 min (or 15 min for rat PFT) with 0.75 μM (0.3 μCi) [^{3}H]FPP and 10–40 nanounits of PFT in a total volume of 20 μl under the standard conditions as above. The reaction was terminated by adding 200 μl of methanol, and the methanol extract was applied onto an Spe+anion exchange column (J.T. Baker) to remove [^{3}H]FPP. The flow-through and methanol washes (200 μl × 6 times) were combined and counted for radioactivity to determine the amount of [^{3}H]farnesylated peptides as described previously [11,20]. For acidic peptides (X = Asp and Glu), the methanol extract was dried in Speed-Vac, and the residue was subjected to analysis by thin layer chromatography on a silica gel 60 plate using a solvent system of 1-propanol/NH_4OH/H_2O (6:3:1, v/v/v) followed by fluorography as described [20].

3. Results and discussion

3.1. Cloning and sequence analysis of *Tc*PFT and *Lmj*PFT

Two allelic forms of the *TcPFT* α-subunit were identified by sequencing multiple PCR amplified clones. These alleles differed at 43 nucleotides and at 27 amino acids. The α-subunits of *Tc*PFT each consist of 628 amino acids, with calculated molecular masses of 72 475 (allele 1) and 72 390 (allele 2) (GenBank accession #s: AF461504 and AF461505). The *Tc*PFT β-subunit consists of 588 amino acids, with calculated molecular mass of 65 807 (GenBank accession #: AF461506). The α- and β-subunits of *Lmj*PFT consist of 764 and 725 amino acids, respectively, with calculated molecular masses of 85 686 and 78 217 (GenBank accession #s: AF461507 and AF461508). Only one allele each was identified for the *TcPFT* β-subunit and for the *LmjPFT* subunits when 4 PCR clones were sequenced. This does not exclude the possibility that other alleles for these genes may be discovered with additional experiments.

The sequence alignments of the α- and β-subunits of *Tb*PFT, *Tc*PFT, and *Lmj*PFT are shown in Fig. 1. The percent identities between pairs of trypanosomatid enzymes are: 47% for *Tb*PFT versus *Tc*PFT, 30% for *Tb*PFT versus *Lmj*PFT, and 32% for *Tc*PFT versus *Lmj*PFT. After alignment of the three trypanosomatid PFTs, the set was aligned to rat PFT, without altering the alignment between the 3 trypanosomatid enzymes, as described previously for the alignment of *Tb*PFT with the rat PFT [11]. Since trypanosomatid PFT contains a number of large insertions compared to the rat enzyme, the crystal structure of rat PFT was used to maximize the alignment of the protein core residues [11] (Fig. 1). In our previous study, it was found that the α-subunit of *Tb*PFT contains 5 inserts of at least 25 amino acids compared to rat PFT. The α-subunits of *Tc*PFT and *Lmj*PFT also contain several inserts, and the insert sizes in *Lmj*PFT are significantly larger than in *Tb*PFT and *Tc*PFT. The *Lmj*PFT α-subunit contains 8 inserts of at least 25 amino acids (labeled αI–αVIII in Fig. 1A). Likewise, the sequence alignment of the β-subunits leads to the identification of 5 inserts of at least 25 amino acids (labeled βI–βV in Fig. 1B) in the *Lmj*PFT β-subunit.

The insert regions that are lacking in rat PFT have relatively low homology between trypanosomatid PFTs. Mapping of these inserts onto the X-ray structure of rat PFT reveals that, without exception, the inserts exist at the surface of the enzyme, in loops between helical secondary structural elements, and away from the active site cavity and the subunit–subunit interface (Fig. 2). The function of these inserts is not known, but the lack of sequence conservation and the topographic location suggests they are not directly involved with the catalytic site of the enzyme. Of the 27 amino acid differences between the two alleles of the α-subunit of *Tc*PFT, 15 residues occur in insert sequences, and none of the residue changes occurs in the catalytic site. Therefore, it is unlikely that the enzymatic properties of the two *Tc*PFT alleles are different.

3.2. Life-cycle expression of *Tc*PFT and *Lmj*PFT

Northern blot analysis shows that *T. cruzi* epimastigotes and trypomastigotes contain detectable mRNAs of both PFT α- and β-subunits. Similarly, *L. major* promastigotes and amastigotes (taken from mouse tissues) contain detectable mRNAs of both PFT α- and β-subunits (data not shown). These data indicate that transcription of *PFT* genes is not limited to particular life-cycle stages of these parasites.

3.3. Expression of recombinant trypanosomatid PFTs in the Sf9/baculovirus system

Recombinant baculoviruses containing *PFT* α- and β-subunit genes of *T. brucei*, *T. cruzi*, or *L. major* were co-infected into Sf9 cells, and cytosolic fractions were assayed for PFT activity using RAS1-CVIM and [^{3}H]FPP as substrates. In all cases, PFT activity was detected at levels about 30- to 50-fold higher than the activity detected in uninfected cells. The results verify that the cloned genes encode functionally active trypanosomatid PFT α- and β-subunits. The recombinant proteins will be useful for enzyme assays to screen inhibitor compounds and for crystallography experiments to determine structures of the enzymes.

3.4. CaaX specificity of trypanosomatid PFTs

We have previously shown that *Tb*PFT displays distinct recognition for the X residue of the CaaX motif in substrates from that of mammalian PFT [2,18]. Substrate specificity of *Tc*PFT and *La*PFT with respect to the X residue was studied using a series of 20 peptides SSCALX (X = one of 20 naturally occurring amino acids) and compared to that of *Tb*PFT and rat PFT. As shown in Fig. 3, *Tc*PFT and *La*PFT display very similar specificity to that of *Tb*PFT. These three trypanosomatid PFTs strongly prefer the peptides containing Met and Gln at the X-position, but not hydrophilic amino acids including Ser, Thr, and Cys, which are good substrates of rat PFT. These CaaX substrate specificity differences are presumably the result of the fact that trypanosomatid PFTs contain residue substitutions compared to mammalian PFT in the enzyme pocket that contacts the X residue. A model of the rat PFT bound to the CaaX substrate Acetyl-Cys-Val-Ile-Met [21], indicates several differences between rat and trypanosomatid PFTs at residues binding the Met of

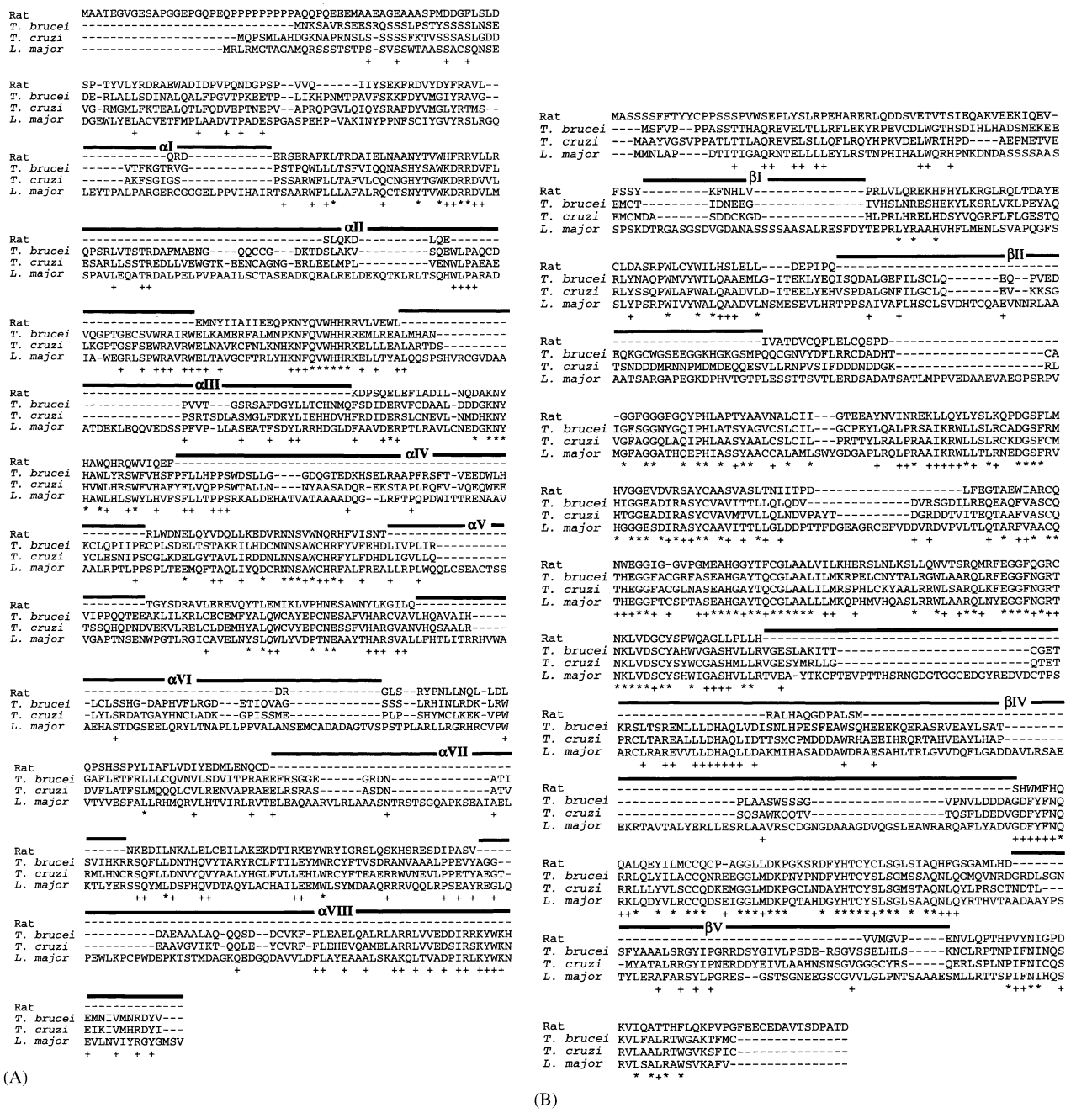

Fig. 1. Amino acid sequence alignment of rat, *Tb*, *Tc*, and *Lmj*PFT α-subunits (panel A) and β-subunits (panel B). The three trypanosomatid PFTs were aligned using the CLUSTAL X program. The alignment of rat PFT and *Tb*PFT was carried out taking account of the X-ray crystal structure of the mammalian enzyme as described previously [11]. Inserts of at least 25 amino acids found in the trypanosomatid enzymes versus rat PFT are indicated by thick lines and numbered sequentially. Residues conserved among all three trypanosomatid enzymes are marked with a plus symbol, and those conserved among all 4 enzymes are marked with an asterisk. (Only allele 1 of *Tc*PFT α-subunit is shown).

the substrate (not shown). In the β-subunit, Ala-98 of rat-PFT is replaced by Asn, Ser, and Pro in *Tb*PFT, *Tc*PFT and *Lmj*PFT, respectively. Ser-99 in rat PFT is replaced by Ala in *Tb*PFT and remains as Ser in *Tc*PFT and *Lmj*PFT. Pro-152 of rat PFT is replaced by Thr, Ala, and Ser in *Tb*PFT, *Tc*PFT and *Lmj*PFT, respectively. In the α-subunit, Ala-129 of the rat enzyme is replaced by Ser, Gly, and Ser in *Tb*PFT, *Tc*PFT and *Lmj*PFT, respectively. The X-ray structures also show that a zinc ion coordinates with Asp-297, Cys-299, and H-362 of rat PFT β-subunit and the cysteine-SH of the CaaX motif [22,23]. These three zinc-binding residues are conserved in PFTs of all species (Fig. 1B).

Site-directed mutagenesis studies of yeast PFT have shown that single-site mutations in the X-residue binding pocket at the position occupied by Pro-152 in the β-

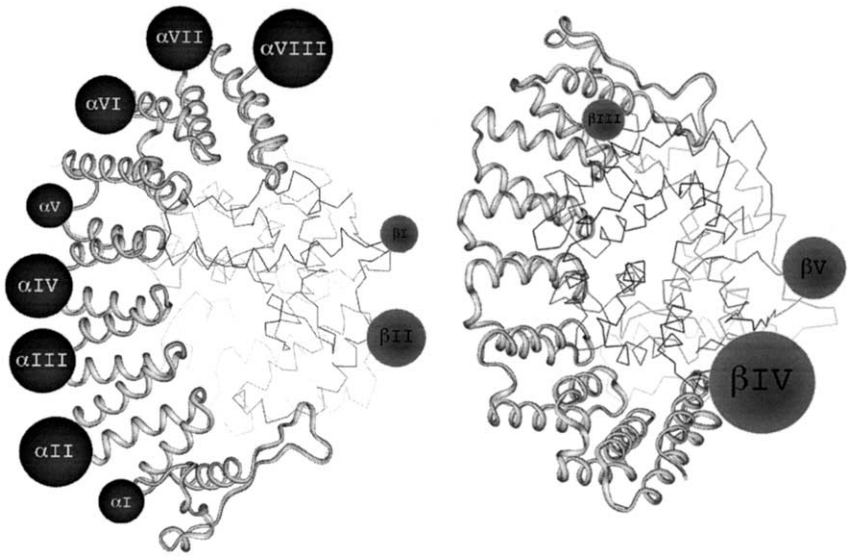

Fig. 2. Positioning of the trypanosomatid inserts onto the X-ray structure of rat-PFT. Inserts of at least 25 amino acids found in the trypanosomatid PFTs versus rat PFT (Fig. 1) are shown as spheres and positioned onto the X-ray structure of rat PFT. The sphere radii are in rank order with the size of the inserts. The α-subunit is shown in ribbon form, while the β-subunit is shown in line form (α-carbon trace). In the left panel, the active site opening is seen as the cavity in the center of the structure formed with elements coming from both subunits. The right panel is generated from the left panel by an approximate 180° rotation about the vertical axis to reveal the location of the βIII, βIV, and βV inserts, which are present on the opposite face of the enzyme as the other loops.

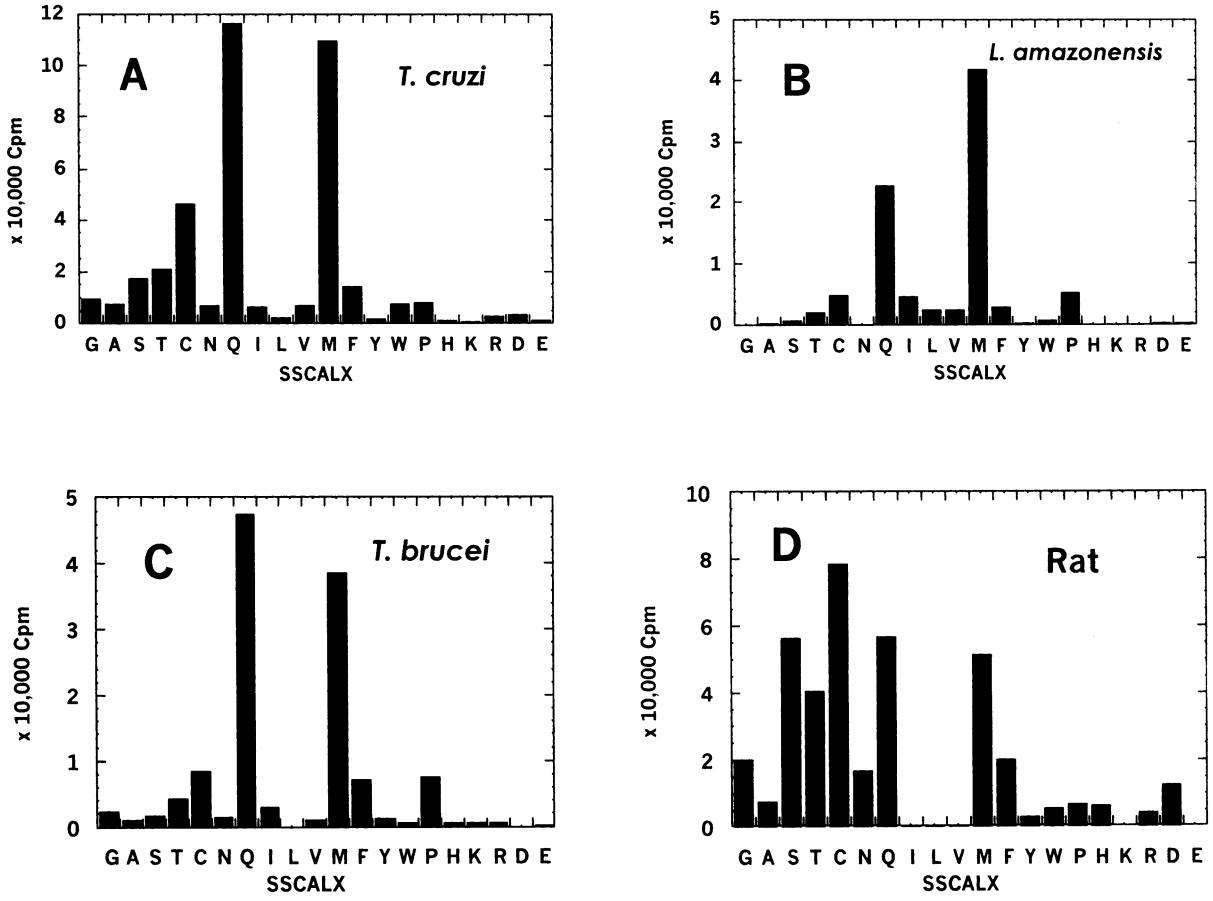

Fig. 3. CAAX specificity of Trypanosomatid PFTs. PFT from the indicated organisms (4–10 nanounits) was incubated with 10 μM each of SSCALX peptides, where X is one of 20 different amino acids, as indicated and 0.75 μM (0.3 μCi) [^{3}H]FPP under the standard assay conditions. Results are expressed as the radioactivity above that for the minus peptide control (6000–10 000 cpm). (A) Native *Tc* PFT (partially purified); (B) Native *La* PFT (partially purified); (C) Native *Tb* PFT [8]; (D) Recombinant rat PFT expressed in the baculovirus/Sf9 cell system.

subunit of rat PFT allow the enzyme to utilize both CIIS and CIIL substrates, whereas the wild type enzyme only farnesylates the CIIS motif, indicating the importance of Pro-152 in the substrate specificity of yeast PFT [24]. *Tb*PFT, *Tc*PFT, and *Lmj*PFT contain Thr, Ala, and Ser, respectively at this position (Pro-152 of rat PFT) (Fig. 3), suggesting possible involvement of this substitution in the altered substrate specificity. However, the a_1 and a_2 residues of the Ca_1a_2X tetrapeptide, in some cases, can make alterations of the X-residue specificity. For example, the *T. cruzi* Rho GTPase that contains the C-terminal CaaX sequence CQLF is well utilized by *Tc*PFT in vitro and is farnesylated in vivo [18] despite the fact that SSCALF is poorly utilized by *Tc*PFT in vitro, shown here (Fig. 3). Thus, additional experiments are required to fully characterize the substrate specificities of trypanosomatid PFTs.

3.5. Implications for design of anti-trypanosomatid agents

A number of CaaX mimetic inhibitors of mammalian PFT contain methionine at the X position [25]. Many of these compounds are also potent inhibitors of trypanosomatid PFTs (unpublished data), presumably because both mammalian and parasite enzymes prefer CaaX substrates with X = methionine. At the same time, based on the clear substrate specificity differences between mammalian and trypanosomatid PFTs revealed in this and earlier studies, we are optimistic that CaaX mimetic inhibitors that are highly selective for parasite PFTs can be obtained by chemical alterations of existing inhibitors of mammalian PFTs. Absolute selectivity toward the parasite enzyme may not be required for PFT inhibitors as anti-parasitic therapeutics since inhibitors of human PFT show therapeutically effective responses in cancer patients when administered at levels below their limiting toxic doses [26]. It will be important to obtain crystal structures of the trypanosomatid PFTs bound to substrates and inhibitors of these enzymes in order to best account for differences in specificity compared to the mammalian PFTs. Inhibitors exploiting differences in the enzyme active sites may have potential to be developed as anti-trypanosomatid chemotherapeutic agents.

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