Available online at www.sciencedirect.com
ScienceDirect
Molecular & Biochemical Parasitology 153 (2007) 186–193
MOLECULAR & BIOCHEMICAL PARASITOLOGY

The *Trypanosoma cruzi PIN1* gene encodes a parvulin peptidyl-prolyl *cis/trans* isomerase able to replace the essential ESS1 in *Saccharomyces cerevisiae*☆

Esteban D. Erben[a], Sebastian Daum[b], María T. Téllez-Iñón[a,*]

[a] *Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET) and Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina*
[b] *Max Planck Research Unit for Enzymology of Protein Folding, Weinbergweg 22, 06120 Halle, Germany*

Received 7 July 2006; received in revised form 3 March 2007; accepted 6 March 2007
Available online 12 March 2007

Abstract

Parvulins are a conserved group of peptidyl-prolyl *cis/trans* isomerases (PPIases) that catalyze the *cis/trans* isomerization of proline-preceding peptide bonds. Parvulin-class PPIases are structurally unrelated to cyclophilins and FK506-binding proteins that are defined as receptors for immunosuppressive drugs. In *Trypanosoma cruzi* we identified parvulin *Tc*PIN1 as a homolog of the human hPin1 PPIase. The 117 amino acids of the *Tc*PIN1 display 40% identity with the catalytic core of hPin1 and exhibit prolyl *cis/trans* isomerase activity. *Tc*PIN1 lacks the WW domain at the N-terminus, and is able to rescue the temperature-sensitive phenotype on a mutation in the *Saccharomyces cerevisiae* hPin1 homolog, ESS1/PTF1. Western blot analysis revealed that the enzyme was present both in dividing and non-dividing forms of *T. cruzi*. In epimastigote cells neither cell growth kinetics nor cell morphology was affected by the overexpression of the small parvulin *Tc*PIN1. These results suggest the occurrence of a supplementary conserved level of post-translational control in trypanosomatids.

Keywords: Prolyl *cis/trans* isomerization; Trypanosomatids; *Trypanosoma cruzi*; Pin1; Parvulin; Cell cycle

1. Introduction

Phosphorylation-directed prolyl isomerization plays a central role in the control of diverse cellular processes. The peptidyl-prolyl *cis/trans* isomerases (PPIases) constitute a conserved family that catalyzes the *cis/trans* isomerization of proline residue-preceding peptide bonds [1,2]. This family consists of different members with diverse substrate specificity and subcellular distribution. Pin1 is a PPIase belonging to the subfamily of parvulins that is distinct from two other well-characterized PPIases families, the cyclophilins and the FK506-binding proteins (FKBPs), which are receptors for immunosuppressive drugs [3–5].

The nuclear parvulin Pin1 has been reported to be essential for the regulation of mitosis because of its interaction with the NIMA kinase pathway, through its ability to bind to and promote phosphorylation-dependent isomerization of phosphoserine (pSer) or phosphothreonine (pThr) proline motifs (pSer/pThr-Pro motifs) [6]. This post-phosphorylation isomerization can lead to conformational changes in the substrate proteins and modulate their functions. Studies in multiple systems including yeast and mammalian cells have suggested that Pin1 is a protein that plays a critical role for mitosis progression [4,6,7]. Depletion of hPin1 in HeLa cells or of its respective homolog ESS1/PTF1 in yeast results in mitotic arrest, whereas overexpression of hPin1 in HeLa cells causes G2 arrest [6,7]. Pin1 participates in the phosphorylation-dependent prolyl isomerization by changing the conformation of its substrates, thus controlling cell cycle progression [8]. It contains two domains, a small N-terminal WW domain implicated in the interaction between proteins, and a C-terminal PPIase catalytic domain [9].

Abbreviations: TFE, trifluoroethanol; PPIase, peptidyl prolyl *cis/trans* isomerase; GFP, green fluorescent protein

☆ The nucleotide sequences reported in this paper have been submitted to the GenBank™ with accession numbers DQ303420 and DQ420359.

* Corresponding author at: INGEBI-CONICET, Vuelta de Obligado 2490, 2nd Floor, 1428 Buenos Aires, Argentina. Tel.: +54 11 4783 2871; fax: +54 11 4786 8578.
E-mail address: mtellez@dna.uba.ar (M.T. Téllez-Iñón).

0166-6851/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2007.03.004

There are four types of WW domain, three of which recognize short proline-rich motifs, and one which recognizes pSer/pThr-Pro motifs [10]. The WW domain of Pin1 recognizes exclusively pSer or pThr proline motifs and most of the known substrates to which Pin1 binds through its WW domain are mitotic phosphoproteins [4,8]. In addition, many proteins that are phosphorylated by CDKs (cyclin-dependent protein kinases) are Pin1 substrates [11–13].

Moreover, Pin1 acts as a transducer of diverse signals and modulates the function of several phosphoproteins involved in regulating different cellular processes including transcription and RNA processing, splicing, proliferation, signaling and DNA damage responses (reviewed in [11]).

Protein phosphorylation modifies the function of proteins to activate a defined set of structural modifications that occur during cell cycle [14]. It has been shown that many of these modifications occur during developmental cycles of parasites [15,16]. Since transcriptional control appears to be relatively irrelevant in trypanosomatids because most genes are transcribed in polycistronic units [17], prolyl isomerization could provide a potent post-translational control. Completion of the *Trypanosoma cruzi* genome database has enabled a broad visualization of genes involved in cell cycle control and life cycle differentiation [18,19]. Trypanosomatids have a broad and complex set of protein kinases including CDKs and mitogen-activated protein kinases, which play important roles in proliferation and cell cycle control [18,20,21]. Interestingly, these kinases phosphorylate proteins exclusively on Ser/Thr residues immediately preceding a proline residue [22,23].

Although most of the identified parvulins of the Pin1-type contain a WW protein–protein interaction domain, all plant enzymes characterized so far lack this module [24–26]. However, it has been demonstrated that even if plant Pin1-type enzymes do not have this domain, they may perform the same function as that of human Pin1 [25–27].

Here, we characterized a small parvulin from the parasite *T. cruzi* for the first time. Like plant homologs, *Tc*PIN1 presents a catalytic core but lacks the WW domain. The protein was equally expressed in the dividing and the non-dividing forms of *T. cruzi*. In epimastigote cells the overexpression of the small parvulin *Tc*PIN1 did not affect the cell growth kinetics of the parasite. Based on functional assays and enzymatic PPIase activity, we showed that *Tc*PIN1 is a member of the Pin1-type PPIases, which suggests that an additional conserved level of post-translational control exists in trypanosomatids.

2. Materials and methods

2.1. Cell cultures and protein preparations

T. cruzi epimastigotes from Tul 2 and CL-Brener strains were cultured as previously described [28]. Metacyclic trypomastigotes were obtained by axenic culture under differentiating conditions. Amastigotes were obtained from Vero cell cultures [29]. Parasite protein extracts were prepared by resuspending the parasite pellets in lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) with protease inhibitors (10 μM E64: l-*trans*-epoxysuccinyl-leucyl amido (4-guanidio) butane, 1 mM phenylmethyl-sulphonyl fluoride, 25 U ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin (Sigma)) followed by three to five freezing and thawing cycles. The complete rupture was confirmed by microscopic visualization. Protein concentration was determined by Bradford method (Bio-Rad Protein Assay).

2.2. Cloning of the hPin1 homolog and its expression in Escherichia coli

The human Pin1 amino acid sequence was used to search for homologous proteins in the *T. cruzi* sequence database at the Institute for Genomic Research (TIGR). The *TcPIN1* coding region was amplified from genomic DNA by PCR using Pfu polymerase (Stratagene, La Jolla, CA). PCR reaction conditions were as follows: initial denaturation cycle at 94 °C for 1 min, followed by 25 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension of 10 min at 72 °C. The primers utilized for PCR cloning of *TcPIN1* gene were TcPin1a 5′-ACGGATCCATGGTGAAGGGTGACTGC-3′ and TcPin1b 5′-ATCGTCGACCTACGCGAGCCGCTTG-3′ as sense and antisense primers respectively, while restriction sites are underlined. The PCR product was inserted into the pQE30 (Qiagen) vector by constructing unique *Bam*HI and *Sal*I sites on the 5′ and 3′ ends of the coding regions. The cloned PCR fragments were sequenced using single primer extension to confirm that no PCR-induced mutations were introduced (Macrogen Inc.).

E. coli JM109 cells (Promega) containing the full pQE30/*TcPIN1* construct were grown at 37 °C in LB medium, supplemented with 100 μg/ml of ampicillin, to a cell density of $A_{600} = 0.7$. Subsequently, expression of the *TcPIN1* gene was induced by addition of 0.4 mM isopropyl β-D-thiogalactoside, and the culture was further grown for 6 h at 37 °C.

2.3. Purification of recombinant TcPIN1

Cells containing the pQE30/*TcPIN1* construct were harvested, resuspended in lysis buffer containing 25 mM Tris/HCl buffer, pH 8.0, 300 mM NaCl, 5% glycerol, 5 mM $MgCl_2$ and 10 mM imidazole, and subjected to seven cycles of sonication (10 pulses of 1 s) followed by 1 min rest between cycles at 4 °C (Heat Systems Ultrasonics, Inc.). The extract was then clarified by centrifugation for 20 min at 15,000 × *g* and the supernatant was loaded on a nickel–nitrilotriacetic acid–agarose affinity resin (Qiagen) previously equilibrated with the same buffer at 4 °C. Protein fractionation was performed according to the manufacturer's instructions. The fractions containing the *Tc*PIN1 fusion protein were pooled, dialyzed overnight against the same buffer without imidazole and re-applied to the Ni–NTA–agarose column. *Tc*PIN1-containing fractions were pooled, dialyzed extensively, and the resulting fractions were used for PPIase activity determinations.

2.4. TcPIN1 antiserum and western blot analysis

In order to raise a *Tc*PIN1 antiserum (anti-*Tc*PIN1), the *TcPIN1* gene was inserted into the pGEX-4T-3 expression

vector to generate the N-terminal translational fusion *Tc*PIN1 protein with *Schistosoma japonicum* glutathione-*S*-transferase (Amersham Biosciences). The purified recombinant protein was used to immunize rabbits as previously described [30]. Protein samples were fractioned on SDS-PAGE gels and transferred electrophoretically to PVDF membranes. The membranes were blocked with 5% powdered milk in Tris/HCl-buffered saline containing 0.05% Tween 20 (TBST-M), and incubated for 1–2 h with anti-*Tc*PIN1, diluted 1:1000 in TBST-M. Blots were washed with TBST and incubated with a 1:5000 dilution of horseradish peroxidase coupled to anti-rabbit secondary antibodies (Amersham Biosciences). Bands were visualized using the ECL Western blotting detection reagents (Amersham Biosciences).

2.5. Northern blot and RT-PCR analysis

Total RNA from the different life cycle stages of *T. cruzi* (epimastigote, trypomastigote, and amastigote) was obtained using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and Northern blotted as previously described [28]. The *Bam*HI/*Sal*I fragment from the pGEX4T-3/*TcPIN1* plasmid, corresponding to the full-length gene was used as a probe. The probe was radiolabeled with [α-^{32}P] dCTP (10^9 cpm pmol^{-1}, NEN) using Prime-a-Gene Labeling System (Promega). Signals were scanned with a phosphorimager Storm 820 (Amersham Pharmacia Biotech) and quantitated using ImageQuant software. First-strand cDNA was synthesized by incubating 4 μg total RNA with M-MLV Reverse Transcriptase (Invitrogen) and oligo(dT)$_{16}$ at 37 °C for 1 h in a 20-μl reaction volume. Subsequently, *TcPIN1* gene was amplified from epimastigote cDNA using the universal splice leader as forward primer and TcPin1b (see Section 2.2) as a gene-specific reverse primer.

2.6. PPIase: peptidyl-prolyl cis/trans isomerase activity assay

PPIase activity assays were performed using a Hewlett-Packard 8453A UV–vis spectrophotometer. The enzyme activity towards the substrates was determined using the protease-free PPIase assay according to Kofron et al. [31] and Janowski et al. [32]. Briefly, 10 nM of *Tc*PIN1 was incubated in 35 mM sodium HEPES buffer, pH 7.8. A 30 mM stock solution of the substrate in 0.5 M LiCl/TFE (anhydrous) was freshly prepared before the measurements. Prior to every measurement, all components except substrate were preincubated for 300 s at 10 °C under vigorous stirring. Each measurement was started after substrate addition (60 μM final substrate concentration) and the *cis*/*trans* isomerization kinetics of the substrate was followed at 330 nm.

2.7. Yeast complementation analysis

The *TcPIN1* sequence was subcloned into the yeast expression vector pJK305-TPI, which allows constitutive expression of the protein under the control of the yeast TPI1 promoter. The temperature-sensitive yeast YGD-ts22W cells (MATa ess1^{H164R} integrated into W303 1A yeast strain) [33] were grown overnight in YPAD medium at 30 °C and were then transformed with *TcPIN1*/pJK305-TPI via electroporation. Transformants were selected growing cells in minimal medium containing 2% glucose but lacking the amino acid leucine, at 30 °C for 2–4 days. Colonies were then restreaked three times and incubated under the same conditions. For the spotting analysis, cells selected on the appropriate medium were resuspended in 10 mM Tris/HCl buffer, pH 7.5, 1 mM EDTA and the density adjusted to $A_{600} = 0.5$. A 10-fold series dilution was performed and 5 μl of each dilution was spotted onto the plates. In order to test for functional complementation, the cells were incubated at permissive (30 °C) and non-permissive (37 °C) temperatures. Cells carrying both the pJK305-TPI vector without the insert and human Pin1 were used as controls. To check for protein expression, cells were grown at 30 °C in minimal medium containing 2% glucose without leucine. The cells were harvested, lysed with glass beads, and total protein extract (50 μg) was separated on a SDS-15% polyacrylamide gel electrophoresis. The expressed proteins were detected by Western blotting using the TcPin1 specific polyclonal antibody (data not shown).

2.8. Overexpression of TcPIN1 in the epimastigote form of T. cruzi

The full-length *TcPIN1*gene was amplified by PCR using primers that had an *Eco*RI site at the 5′ end and a *Sal*I site at the 3′ end of the gene. PCR-amplified *TcPIN1* was cloned into the *Eco*RI/*Sal*I digested pTREX vector. *T. cruzi* epimastigote cells of the CL Brener strain were transfected with the plasmid as previously described [34]. Complete selection confirmed by the total GFP positive control population, was obtained after 2 months in the presence of the antibiotic G418 (Invitrogen).

3. Results

3.1. Presence of Pin1-type PPIase gene in trypanosomatids

A systematic homolog search for a Pin1 candidate in the *T. cruzi* database was performed using the described human Pin1 sequence without success. However, when the catalytic PPIase domain was used, potential candidates resembling the plant Pin1-type PPIases [24–26] were obtained. The complete *TcPIN1* coding sequence (354-bp long) encodes a 117-amino-acid protein, with a predicted molecular weight of approximately 13 kDa. A search in the *Trypanosoma brucei* and *Leishmania major* genome databases revealed that putative parvulins Pin1-type PPIases homologs are present in all trypanosomatids with a high degree of identity. An alignment of *TcPIN1* with orthologs from several organisms is shown in Fig. 1.

*Tc*PIN1 has 40% amino acid sequence identity with the PPIase domain of human Pin1 and 45% with the *Arabidopsis thaliana* homolog. Moreover, the amino acid insertion observed in *Tc*PIN1 (residues Thr^{31} and Ala^{32}) is also observed in both *At*Pin1 (four residues Lys^{31} to Leu^{34}) and other plant Pin1-type PPIases [25–27], as well as in *E. coli* protein (Fig. 1A). As all plant Pin1-type PPIases characterized so far, *Tc*PIN1 displays a PPIase catalytic domain but not an N-terminal

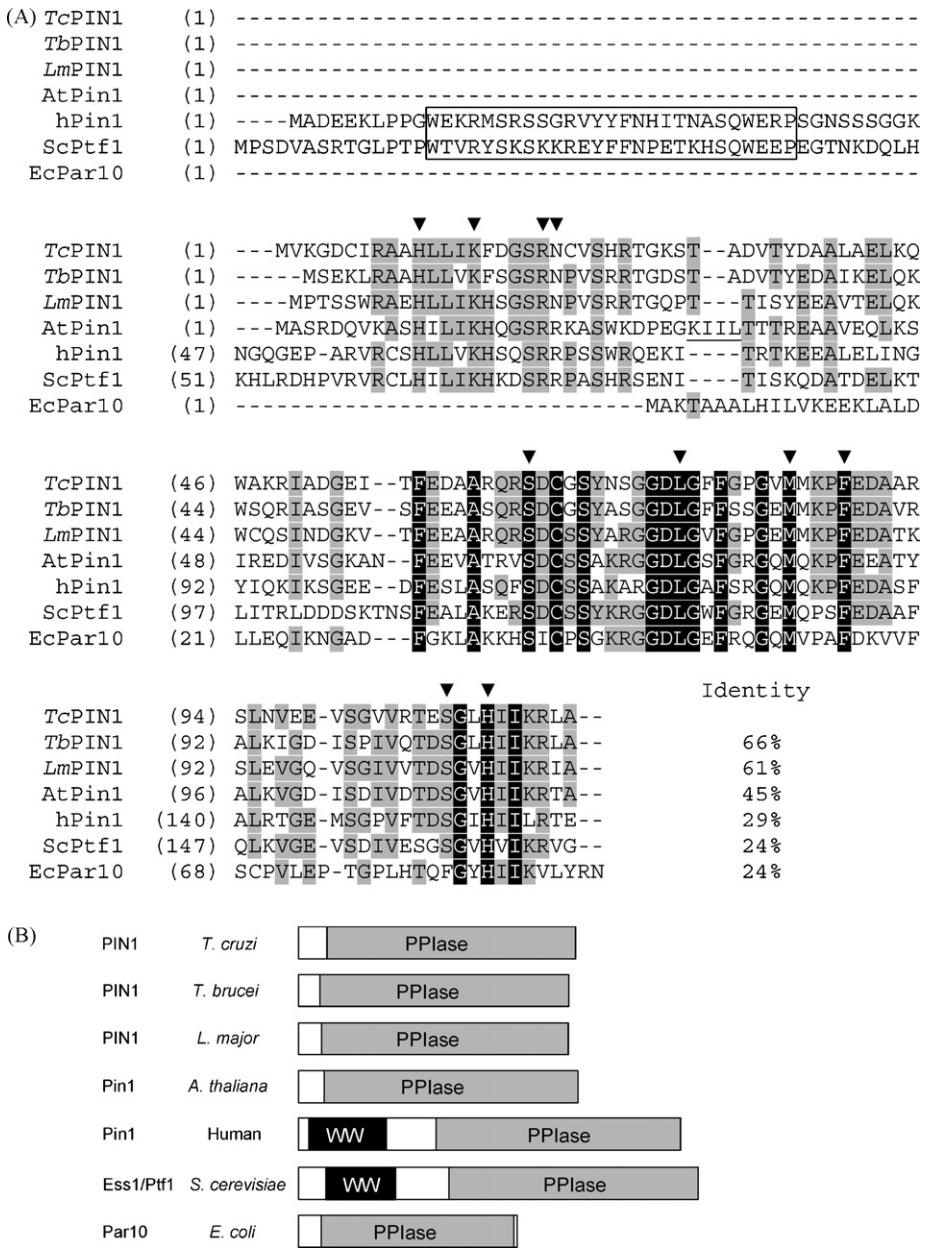

Fig. 1. (A) Sequence alignment of some members of the parvulin family of PPIases. Identical (black) or conservatively substituted (gray) residues in at least 50% of the sequences are highlighted. Degrees of identity are shown to the right of each sequence. The four-residue insertion in the plant protein is *underlined*. The core of the N-terminal WW module of the proteins is *boxed* and residues shown to be implicated in the substrate recognition are indicated by black triangles [40]. *Tc*PIN1 is from *T. cruzi* (DQ303420), *Tb*PIN1 is from *T. brucei* (AAX69357), *Lm*PIN1 is from *L. major* (CAJ07069), AtPin1 is from *A. thaliana* (AAD20122), hPin1 is from humans (AAC50492), ScPtf1 is from *S. cerevisiae* (CAA59961) and EcPar10 is from *E. coli* (S48658). (B) Schematic representation of the Pin primary domain structure of selected Pin1 proteins from different species.

WW protein–protein binding domain or an analogous module. Besides, prokaryotic parvulins with a rather unspecific substrate recognition pattern like that of Par10 from *E. coli* display less sequence identity (24%). *Tc*PIN1 is the first *T. cruzi* Pin1-type PPIase homolog identified and characterized in trypanosomatids. A scheme of these PPIases is shown in Fig. 1B.

3.2. PPIase activity

The PPIase activity of the recombinant *Tc*PIN1 enzyme was determined by using the protease-free assay. Under these conditions, the substrate specificity of the native recombinant *Tc*PIN1 toward the substrate succinyl-Ala-Glu-Pro-Phe-*p*-nitroanilide resulted in a K_{cat}/K_m value of 3.97 ($\pm$0.24) $\times 10^5\ M^{-1}\ s^{-1}$, whereas isomerase activity for the substrate containing Ala-Pro bonds exhibited lower activity (1.54 ($\pm$0.14) $\times 10^4\ M^{-1}\ s^{-1}$). The values of the catalytic efficiencies showed a considerable decrease when compared to other Pin1-related parvulins with the same substrates. The catalytic efficiencies of hPin1 determined in the same experiment showed values of $3.53 \times 10^6\ M^{-1}\ s^{-1}$ and $4.84 \times 10^4\ M^{-1}\ s^{-1}$, respectively. Remarkably, despite the high sequence identity of the PPIases active sites, *Tc*PIN1

displayed a catalytic efficiency nine-fold lower toward the substrates tested than that determined for hPin1 and plant-related PPIases [24,26].

These results indicate that *Tc*PIN1 is active as a PPIase enzyme and could suggest that this parvulin prefers peptides with a negatively charged glutamyl instead of an alanyl residue N-terminal to the prolyl bonds.

3.3. *TcPIN1 is expressed throughout the life cycle of the parasite*

The expression pattern of *T. cruzi PIN1* gene was studied in all life cycle stages of the parasite using the full-length *TcPIN1* as a probe. As seen in Fig. 2A, *TcPIN1* mRNA was detected in all forms examined although in dissimilar amounts. Whereas two transcripts were visualized in epimastigote stage, just a single band was observed in amastigote and trypomastigote forms. To explore the specificity of this extra epimastigote fragment band, we performed an RT-PCR assay under conditions described in Section 2.5. Two DNA fragments of 461 bp and 556 bp, respectively, corresponding to the region between *TcPIN1* end coding sequence and the splice leader acceptor site at the 5′ untranslated region (UTR) were amplified in epimastigote stage. No amplification signal was detected in the negative control (data not shown). Nucleotide sequence analysis of the RT-PCR products revealed that both transcripts share the same ORF but different 5′ untranslated region, and that both long and short UTRs share the same sequence from nucleotide-1 to the mini-exon addition site of the short 5′ UTR. This analysis showed that the difference in length is due to the use of ambivalent AG dinucleotides as mini-exon addition sites.

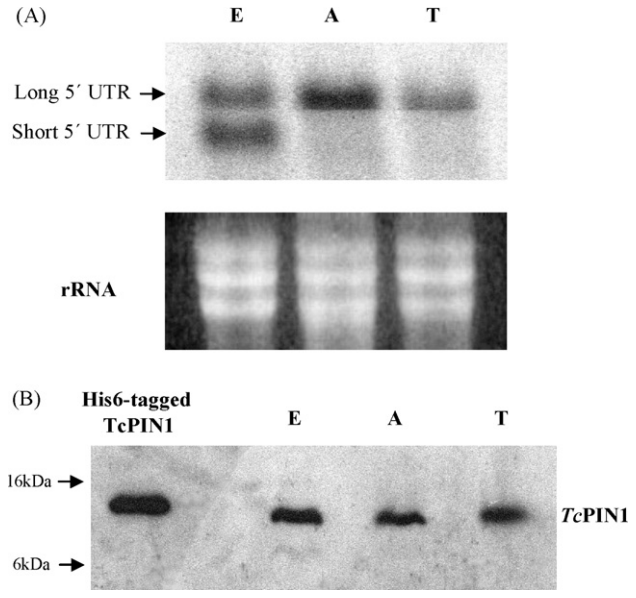

Fig. 2. (A) Association of *TcPIN1* mRNA expression with cell differentiation in *T. cruzi* parasite. *TcPIN1* mRNA was identified in all forms examined (upper panel). Total RNA from *T. cruzi* epimastigote (E), amastigote (A) and trypomastigote (T) forms (30 μg each) was separated on 2% formaldehyde-agarose gels, transferred to nylon membranes and hybridized with *TcPIN1* as indicated. Two transcripts were visualized in the epimastigote stage (long and short 5′ UTR) and a single band was observed in amastigote and trypomastigote forms (long 5′ UTR). Ethidium bromide staining of rRNA was used as loading control (lower panel). (B) *Tc*PIN1 is expressed in all three stages of the parasite. Protein extracts from epimastigote (E), amastigote (A) and trypomastigote (T) forms (75 μg each) were separated by 15% SDS-PAGE gel and electroblotted on PVDF membranes. His-tagged recombinant *Tc*PIN1 (100 ng) was used as a positive control (lane 1). Blots were probed with anti *Tc*PIN1 and with pre-immune serum (data not shown). The position of 13 kDa *Tc*PIN1 is indicated.

To determine the levels of *Tc*PIN1 protein expression we performed a Western blot assay using the specific antiserum tested on blots containing recombinant *Tc*PIN1 and *T. cruzi* extracts. As it can be seen in Fig. 2B, the anti-*Tc*PIN1 antiserum recognizes a single 13-kDa protein in total cell extracts in all three life cycle stages of the parasite. No prominent differences in *Tc*PIN1 protein expression can be observed between the three forms and no other bands were detected in cells extract. The pre-immune serum was used as a negative control (data not shown). Nevertheless, there was not a clear correspondence between the mRNA detected and the endogenous parasite PIN1 protein levels, possibly related to post-transcriptional regulation processes not yet sufficiently explained in trypanosomatids [35].

3.4. *Yeast complementation analysis*

It has been previously shown that hPin1 and other PPIases-related parvulins of plants clearly replace the Ess1/Ptf1 protein by preventing terminal mitotic arrest of cells under restrictive temperatures [6,24,26]. Since there is no genetic approach yet available in *T. cruzi*, the rescuing of *S. cerevisiae* ESS1 conditional mutant by recombinant *T. cruzi* PPIase was tested. This strain, which grows normally at 30 °C (permissive temperature), arrests at G2/M transition after two to three generations at 37 °C (restrictive temperature) [7,33,36]. The complete *TcPIN1* gene was subcloned in the *S. cerevisiae* expression vector pJK305-TPI and transformed in the budding yeast ESS1 mutant. After selection on leucine-deficient minimal medium plates, only colonies transformed by the pJK305-TPI containing the *TcPIN1* gene grew at the restrictive temperature, whereas the colonies transfected with the empty vector grew poorly (Fig. 3). In the same set of experiments, human Pin1 was used as a positive control. In contrast, the temperature sensitivity of the yeast cells was not abolished by an hPar14 *T. cruzi* homolog (not shown) (GenBank™ accession number DQ420359). Human Par14, a second member of the parvulin family of PPIases, does not catalyze the isomerization of phosphorylated Ser/Thr-Pro bonds but shows strong preference for peptide substrates containing arginine side chains preceding proline [37]. *Tc*PIN1 was able to rescue ESS1^{ts} conditional mutant and these results suggest that this parvulin, despite lacking the WW domain, maintains a phosphorylation-directed prolyl isomerization activity.

3.5. *Overexpression of TcPIN1 in T. cruzi epimastigotes*

In order to analyze the effect of *Tc*PIN1 overexpression, we cloned the full-length *TcPIN1* gene into the high constitutive expression vector pTREX [34]. The CL Brener strain was transformed with either a vector containing GFP or a vector encoding

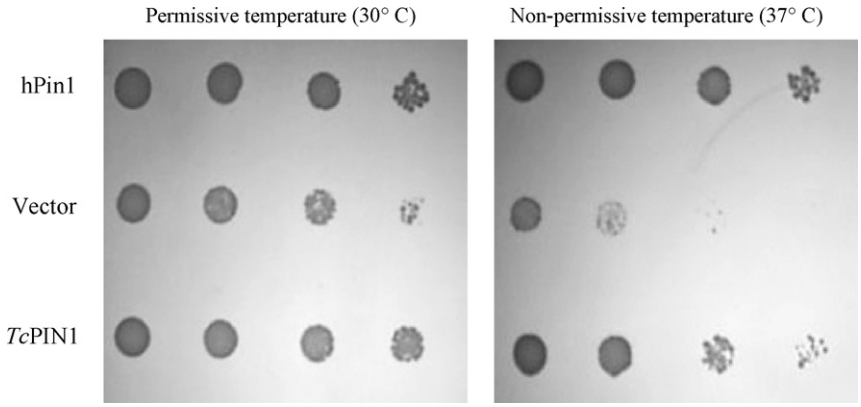

Fig. 3. Rescue of *S. cerevisiae* ESS1/PTF1 gene conditional mutant strain. Petri dishes were spotted with 10-fold serial dilutions of YGD-ts22W cells transformed with *TcPIN1* expression construct (bottom). The plates were incubated at 30 °C (permissive temperature) or 37 °C (restrictive temperature) for 2–4 days. Cells expressing hPin1 (upper) or the empty vector (middle) were used as positive and negative controls, respectively.

TcPIN1 to generate control and overexpression strains, respectively.

After 6 days of culture, Northern blot analysis of stable pTREX/*TcPIN1* transfected cells showed a notably enhanced level of *TcPIN1* (Fig. 4, upper inset). Also, there was a clear correspondence between mRNA and parasite PIN1 enzyme levels. These levels were more than 50-fold higher in the pTREX-transfected populations when compared with the control cells corresponding to the same culture day (Fig. 4, upper and lower inset). Under the conditions tested, these observations suggest that *Tc*PIN1 overexpression did not modify the cell growth kinetics. Moreover, no morphological differences were observed between the stable transfected cells, the non-transfected parasites and the GFP-expressing populations (data not shown). Also, overexpressing cells analyzed by the numbers of nuclei and kinetoplasts did not display any changes.

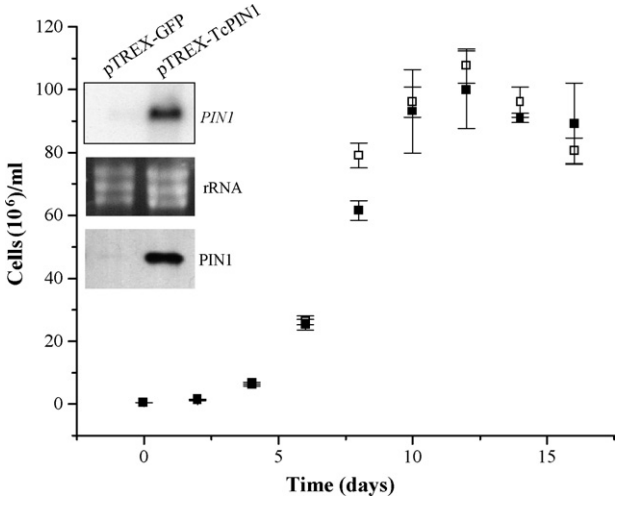

Fig. 4. Effect of overexpression of *Tc*PIN1 on the epimastigote form growth *in vitro*. Cell samples were collected after 6 days of culture (mid-log-phase; 5×10^5 parasites). Total RNA (5 μg) and proteins were analyzed and quantitated by Northern (top inset) and Western blots (bottom inset). Cell growth was plotted vs. time. No significant differences were observed between the transfected parasites (□) and the GFP-expressing populations (■).

4. Discussion

In this work, we report the identification of *Tc*PIN1, a novel small parvulin, and show evidence indicating that this peptidyl-prolyl *cis/trans* isomerase could be the human Pin1 homolog in *T. cruzi.*

Human Pin1 is a phosphorylation-dependent prolyl isomerase that specifically isomerizes the phosphorylated Ser/Thr-proline bonds and its substrate specificity results from the organization of the Xaa-Pro binding pocket [39]. Its three-dimensional crystal structure shows that hPin1 exhibits at this site a cluster of basic residues composed of Lys-63, Arg-68 and Arg-69, which mediates the selectivity for the side chain N-terminal to proline. In contrast, in cyclophilins and FKBPs, a hydrophobic pocket containing aromatic and aliphatic residues sequesters the aliphatic Pro side chain thus determining the different PPIases substrate specificity [40].

In *Tc*PIN1, these specific sequence features exist as Lys-15, Arg-20 and Asn-21, respectively. As it can be seen in Fig. 1, *Tc*PIN1 contains an Asn residue instead of Arg and this sequence motif seems to be a characteristic of the trypanosomatid parvulins. On the other hand, most of the residues proposed to be implicated in forming the binding site for the cyclic side chain of the proline and the residues whose side chain surrounds the peptidyl-prolyl bond in hPin1 are also present in *Tc*PIN1 (Fig. 1) [39].

Considering the high degree of sequence identity of the active sites of the PPIases, an interesting question is why *Tc*PIN1 displays differences in its enzymatic activity.

In plants, three hPin1 homologs have been characterized [24–26]. These enzymes show the same specificity for pSer/pThr motifs as hPin1, but like *Tc*PIN1, they do not have the WW domain at the N-terminus (Fig. 1). Although all plant Pin1-type PPIases present a four-residue insertion, *T. cruzi* parvulin presents only two amino acids (Fig. 1, residues Thr^{31} and Ala^{32}). This region, next to the phospho-specific recognition site, corresponds to a loop between strand β1 and α1 helix in the structure of the *A. thaliana* Pin1, and is predicted to be implicated in the interaction between the enzyme and its substrates [27]. Further-

more, by removing these four extra residues, the plant Pin1-type PPIases are unable to rescue the ESS1^{ts} phenotype of *S. cerevisiae* [26]. From our results, we can speculate that this region, where the homology between human, plant and trypanosomatid PPIases is the lowest, is responsible for *Tc*PIN1 low level of prolyl isomerization activity (Fig. 1A). Conversely, since the *T. cruzi* genome is fully sequenced, the possibility that the WW domain-containing Pin1-type PPIase is identifiable can be ruled out. However, the chance that the low activity observed could be due to improper folding of *Tc*PIN1 cannot be excluded. Nevertheless, this result might not hold true for its endogenous substrates.

Here, we showed that parasite *Tc*PIN1, like human Pin1 and its plant homologs [24,26], can rescue the yeast strain YGD-ts22W at the non-permissive temperature (Fig. 3). In contrast, an hPar14 *T. cruzi* homolog is not able to rescue this yeast strain (unpublished results). Even though results from complementation experiments in yeast do not represent unequivocal evidence that *Tc*PIN1 in *T. cruzi* has the same function as Ess1/Ptf1 in yeast, they suggest that despite its low level of isomerization activity, *Tc*PIN1 functions like full-length hPin1 (at least in *S. cerevisiae*), suggesting a phosphorylation-directed prolyl isomerization activity in trypanosomes. Furthermore, our work is in agreement with the reports by Gemmill et al. [36], who have shown that low levels of ESS1 are sufficient to support budding yeast growth *in vivo*. Nevertheless, experimental results by Yaffe et al. [4] have demonstrated that the incorporation of negatively charged side chains of Glu (or Asp) immediately preceding a Pro residue, which could mimic the phosphorylated residue (pSer/pThr-Pro motifs), results in significant increase of PPIase activity of Pin1. Taken together, these results suggest that phosphorylation-specific PPIases are functionally conserved in yeast, mammalian, plant and ancient trypanosomatid cells.

Cell cycle functional analyses indicate that Pin1 is specifically required for mitosis control [6]. During the cell cycle, Pin1 concentration is constant, but Pin1-binding proteins such as Cdc25, Wee1, Plk1, NiMA and Cdc27 are highly regulated (reviewed in [41]). Antibodies raised against *Tc*PIN1 showed that the PPIase enzyme was present in all three stages of the parasite (Fig. 2B).

It is worth pointing out that Yao and coworkers [26] found that expression of Pin1 from *Malus domestica* (MdPin1) is closely associated with cell division and suggested that MdPin1 probably plays a role in cell cycle progression in apples, as does Pin1 in vertebrate and yeast cells. Interestingly, despite the high similarity that these small parvulins display (42% amino acid sequence identity), *Tc*PIN1 is equally expressed in both the proliferative and non-proliferative forms of the parasite. Moreover, as shown in this work, in epimastigote cells neither the cell growth kinetics (Fig. 4) nor cell morphology (data not shown) was affected by the overexpression of the small parvulin *Tc*PIN1. These observations are unique features that distinguish *Tc*PIN1 from all the Pin1-type PPIases described so far. Additionally, we did not localize *Tc*PIN1 in nuclei (unpublished results) in contrast to that observed with hPin1 and its homologs from diverse other organisms [6,24,38].

Even though the specific function of this small PPIase is yet to be determined, here we showed that *Tc*PIN1 could belong to the parvulin Pin1-type PPIase. Furthermore, small parvulin Pin1-type PPIases lacking the WW domain appear to be a common evolutionary specialization of plants and trypanosomatids. We demonstrated that the parasite enzyme can rescue the lethal phenotype of a mutation in the hPin1 homolog ESS1/PTF1 gene in *S. cerevisiae* and exhibits PPIase activity toward specific substrates. However, the fact that under the conditions used in this experiment the overexpression of *Tc*PIN1 does not alter the parasite cell cycle indicates that the genuine biological function of the protein is yet to be elucidated. Future studies in this direction are necessary to identify the binding proteins/substrates for *Tc*PIN1 and we are currently engaged in this task.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), the National Research Council (CONICET) and the Universidad de Buenos Aires to MTTI. We are indebted to Dr. Berta F. de Cazzulo and Dr. Claudio Pereira for providing amastigote and trypomastigote cultures. We are grateful to Dr. G. Fischer for helpful support. Also we thank Steven D. Hanes (Wadsworth Center) for the yeast plasmids and strains. EE is a Doctoral Fellow of ANPCYT; MTTI is a Career Investigator of CONICET.

References

[1] Hunter T. Prolyl isomerases and nuclear function. Cell 1998;92:141–3.
[2] Schmid FX. Protein folding prolyl isomerases join the fold. Curr Biol 1995;5:993–4.
[3] Fischer G, Aumuller T. Regulation of peptide bond *cis/trans* isomerization by enzyme catalysis and its implication in physiological processes. Rev Physiol Biochem Pharmacol 2003;148:105–50.
[4] Yaffe MB, Schutkowski M, Shen M, et al. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science 1997;278:1957–60.
[5] Schreiber SL. Chemistry and biology of the immunophilins and their immunosuppresive ligands. Science 1991;251:283–7.
[6] Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature 1996;380:544–7.
[7] Hanes SD, Shank PR, Bostian KA. Sequence and mutational analysis of ESS1, a gene essential for growth in *Saccharomyces cerevisiae*. Yeast 1989;5:55–72.
[8] Shen M, Stukenberg PT, Kirschner MW, Lu KP. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. Genes Dev 1998;12:706–20.
[9] Lu PJ, Zhou XZ, Shen M, Lu KP. Function of WW domains as phosphoserine- or phosphothreonine-binding modules. Science 1999;283:1325–8.
[10] Sudol M, Sliwa K, Russo T. Functions of WW domains in the nucleus. FEBS Lett 2001;490:190–5.
[11] Lu KP. Pinning down cell signaling, cancer and Alzheimer's disease. Trends Biochem Sci 2004;29:200–9.
[12] Xu YX, Hirose Y, Zhou XZ, Lu KP, Manley JL. Pin1 modulates the structure and function of human RNA polymerase II. Genes Dev 2003;17:2765–76.
[13] Palancade B, Marshall NF, Tremeau-Bravard A, Bensaude O, Dahmus ME, Dubois MF. Dephosphorylation of RNA polymerase II by CTD-phosphatase FCP1 is inhibited by phospho-CTD associating proteins. J Mol Biol 2004;335:415–24.

[14] Lew D, Kornbluth S. Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr Opin Cell Biol 1996;8:795–804.
[15] Parsons M, Valentini M, Carter V. Protein kinases in divergent eukaryotes: identification of protein kinase activities regulated during trypanosome development. Proc Natl Acad Sci USA 1993;90:2656–60.
[16] Dell K, Engel J. Stage-specific regulation of protein phosphorylation in *Leishmania major*. Mol Biochem Parasitol 1994;64:283–92.
[17] Clayton C. Developmental regulation of nuclear gene expression in *Trypanosoma brucei*. Prog Nucleic Acid Res Mol Biol 1992;43:37–66.
[18] Parsons M, Worthey E, Ward P, Mottram J. Comparative analysis of the kinomes of three pathogenic trypanosomatids: *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*. BMC Genomics 2005;6:127.
[19] El-Sayed N, Myler P, Bartholomeu D, et al. The genome sequence of *Trypanosoma cruzi* etiologic agent of chagas disease. Science 2005;309:409–15.
[20] Doerig C, Chakrabarti D, Kappes B, Matthews K. The cell cycle in protozoan parasites. Prog Cell Cycle Res 2000;4:163–83.
[21] Hammarton T, Mottram J, Doerig C. The cell cycle of parasitic protozoa: potential for chemotherapeutic exploitation. Prog Cell Cycle Res 2003;5:91–101.
[22] Nigg EA. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. BioEssays 1995;17:471–80.
[23] Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem 1995;270:14843–6.
[24] Metzner M, Stoller G, Rucknagel KP, et al. Functional replacement of the essential ESS1 in yeast by the plant parvulin DlPar13. J Biol Chem 2001;276:13524–9.
[25] Landrieu I, De Veylder L, Fruchart JS, et al. The *Arabidopsis thaliana* PIN1At gene encodes a single-domain phosphorylation-dependent peptidyl prolyl *cis*/*trans* isomerase. J Biol Chem 2000;275:10577–81.
[26] Yao JL, Kops O, Lu PJ, Lu KP. Functional conservation of phosphorylation-specific prolyl isomerases in plants. J Biol Chem 2001;276:13517–23.
[27] Landrieu I, Wieruszeski JM, Wintjens R, Inze D, Lippens G. Solution structure of the single-domain prolyl *cis*/*trans* isomerase PIN1At from *Arabidopsis thaliana*. J Mol Biol 2002;320:321–32.
[28] Gómez EB, Kornblihtt AR, Tellez-Iñón MT. Cloning of a cdc2-related protein kinase from *Trypanosoma cruzi* that interacts with mammalian cyclins. Mol Biochem Parasitol 1998;91:337–51.
[29] Andrews N, Colli W. Adhesion and interiorization of *Trypanosoma cruzi* in mammalian cells. J Protozool 1982;29:264–9.
[30] Santori MI, Laria S, Gómez EB, Espinosa I, Galanti N, Tellez-Iñón MT. Evidence for CRK3 participation in the cell division cycle of *Trypanosoma cruzi*. Mol Biochem Parasitol 2002;121:225–32.
[31] Kofron JL, Kuzmic P, Kishore V, Colon-Bonilla E, Rich DH. Determination of kinetic constants for peptidyl prolyl *cis–trans* isomerases by an improved spectrophotometric assay. Biochemistry 1991;30:6127–34.
[32] Janowski B, Wollner S, Schutkowski M, Fischer G. A protease-free assay for peptidyl prolyl *cis*/*trans* isomerases using standard peptide substrates. Anal Biochem 1997;252:299–307.
[33] Wu X, Rossettini A, Hanes SD. The ESS1 prolyl isomerase and its suppressor BYE1 interact with RNA pol II to inhibit transcription elongation in *Saccharomyces cerevisiae*. Genetics 2003;165:1687–702.
[34] Vázquez MP, Levin MJ. Functional analysis of the intergenic regions of TcP2b gene loci allowed the construction of an improved *Trypanosoma cruzi* expression vector. Gene 1999;239:217–25.
[35] Clayton C. Life without transcriptional control? From fly to man and back again. EMBO J 2002;21:1881–8.
[36] Gemmill TR, Wu X, Hanes SD. Vanishingly low levels of Ess1 prolyl-isomerase activity are sufficient for growth in *Saccharomyces cerevisiae*. J Biol Chem 2005;280:15510–7.
[37] Uchida T, Fujimori F, Tradler T, Fischer G, Rahfeld JU. Identification and characterization of a 14 kDa human protein as a novel parvulin-like peptidyl prolyl *cis*/*trans* isomerase. FEBS Lett 1999;446:278–82.
[38] Huang HK, Forsburg SL, John UP, O'Connell MJ, Hunter T. Isolation and characterization of the Pin1/Ess1p homologue in *Schizosaccharomyces pombe*. J Cell Sci 2001;114:3779–88.
[39] Ranganathan R, Lu KP, Hunter T, Noel JP. Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. Cell 1997;89:875–86.
[40] Fanghanel J, Fischer G. Insights into the catalytic mechanism of peptidyl prolyl *cis*/*trans* isomerases. Front Biosci 2004;9:3453–78.
[41] Lu KP. Phosphorylation-dependent prolyl isomerization: a novel cell cycle regulatory mechanism. Prog Cell Cycle Res 2000;4:83–96.