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Biochemical characterization of a protein tyrosine phosphatase from *Trypanosoma cruzi* involved in metacyclogenesis and cell invasion

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ABSTRACT

Protein tyrosine phosphatases (PTPs) form a large family of enzymes involved in the regulation of numerous cellular functions in eukaryotes. Several protein tyrosine phosphatases have been recently identified in trypanosomatides. Here we report the purification and biochemical characterization of TcPTP1, a protein tyrosine phosphatase from *Trypanosoma cruzi*, the causing agent of Chagas' disease. The enzyme was cloned and expressed recombinantly in *Escherichia coli* and purified by Ni-affinity chromatography. Biochemical characterization of recombinant TcPTP1 with the PTP pseudo-substrate pNPP allowed the estimation of a Michaelis–Menten constant K_m of 4.5 mM and a k_{cat} of 2.8 s^{-1}. We were able to demonstrate inhibition of the enzyme by the PTP1b inhibitor BZ3, which on its turn was able to accelerate the differentiation of epimastigotes into metacyclic forms of *T. cruzi* induced by nutritional stress. Additionally, this compound was able to inhibit by 50% the infectivity of *T. cruzi* trypomastigotes in a separate cellular assay. In conclusion our results indicate that TcPTP1 is of importance for cellular differentiation and invasivity of this parasite and thus is a valid target for the rational drug design of potential antibiotics directed against *T. cruzi*.

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1. Introduction

Protein tyrosine phosphatases (PTPs) form a large family of proteins in eukaryotic genomes with a wide range of functions [1]. Solution of the structures of several PTPs has established an enzymatic mechanism where a catalytic cysteine from the core catalytic motif (H/V)C(X)$_5$R(S/T) (Supplementary Fig. S1) acts as a nucleophile to form a covalent thiophosphate intermediate with the substrate. This intermediate is additionally stabilized by a catalytic arginine and hydrolyzed with the help of a catalytic aspartate, which functions as a general acid/base [1,2].

Trypanosoma cruzi is the causing agent of Chagas' disease, which is estimated to affect 8–15 million people in Latin America leading to up to 20 thousand deaths yearly [3,4]. Eighty-six phosphatases have been identified in the genome of *T. cruzi*, two of which show signatures of PTPs [5]. These two have been termed TcPTP1 and TcPTP2 and show 15% identity with *Homo sapiens* PTP1B [6]. TcPTP1 furthermore shows 62% identity with *Trypanosoma brucei* PTP1 (TbPTP1; Supplementary Fig. S1) [7] which has been recently shown to arrest the trypomastigote form of this parasite in the so called "stumpy" form [8,9] and whose structure has been recently solved [10].

Only two drugs (nifurtimox and benznidazole; [11]) are currently available for the treatment of Chagas' disease both showing severe side-effects and/or a low overall efficacy [12]. Due to the potential important function of PTPs in the trypanosomatids cell cycle, and the interest in the field in developing drugs against related PTPs from humans such as PTP1B [6] and Shp2 [13], these proteins constitute an interesting target for drug development. Here we provide the first step to these approaches by cloning and characterizing PTP1 from *T. cruzi*.

Abbreviations: PTP, protein tyrosine phosphatase; pNPP, *p*-nitrophenylphosphate; BZ3, 3-(3,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonic-acid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide.

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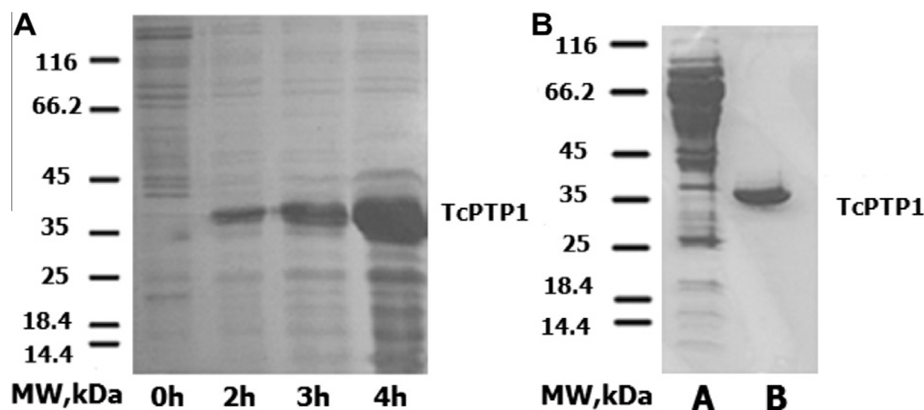

Fig. 1. Recombinant TcPTP1 expression. (A) Test expression after induction with 1 mM IPTG at 30 °C. (B) TcPTP1 affinity purification. Lane A: Total protein; lane B: Purified protein.

2. Materials and methods

2.1. Construct

The protein tyrosine phosphatase TcPTP1 (GI:3554415 Tc00.1047053510187.234) from *T. cruzi* (CL Brener strain) was amplified by PCR using the oligonucleotides 5′-ggccggagatctatgaatgattcgaactgc and 5′-ggccggggtacccctacctgctattcaacagaccg as primers and inserted into the *Bam*HI and *Kpn*I sites of the pQtev [14] expression vector, leading to an expression construct with an N-terminal His-tag. The construct was verified by sequencing using a Hitachi 3130xl Genetic Analyzer (Applied Biosystem) sequencer.

2.2. Protein purification

TcPTP1 was expressed as a 36.8 kDa His-tagged protein after 4 h induction (30 °C) with 1 mM IPTG. Protein was purified after cell lysis using a French Press in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol buffer using Ni-affinity chromatography (GE-Healthcare), dialyzed with 50 mM Tris–HCl pH 7.5, 3 mM DTT and concentrated to 10 mg/mL.

2.3. Protein analytics

Protein was analyzed using SDS–PAGE [15] and quantified using the Bradford method [16].

2.4. Activities assay (kinetics parameters)

Protein activity was determined by Michaelis–Menten steady state kinetics using the PTP pseudo-substrate pNPP (Sigma–Aldrich) at different concentrations in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 3 mM DTT at room temperature with a Magellan™ plate reader (TECAN). Absorption of product (*p*-nitrophenol) was measured as optical density at 405 nm. Data were fitted using non-linear regression analysis using the GraphPad Prism software package. k_{cat} was calculated from $V_{\max} = k_{cat} \cdot [E_{tot}]$ and $V_{\max} = d(\mathrm{OD}_{405})/dt \cdot \varepsilon^{-1} \cdot d_{abs}^{-1} \cdot \varepsilon$, the molar absorption coefficient of *p*-nitrophenol at 405 nm was assumed to be 18,450 M^{-1} cm^{-1}. Error of measurement was calculated from several independent measurements and is given as SEM.

2.5. Metacyclogenesis

The *T. cruzi* Dm28c strain was cultured in liver infusion tryptose (LIT) medium, containing 10% fetal bovine serum at 28 °C [17]. To

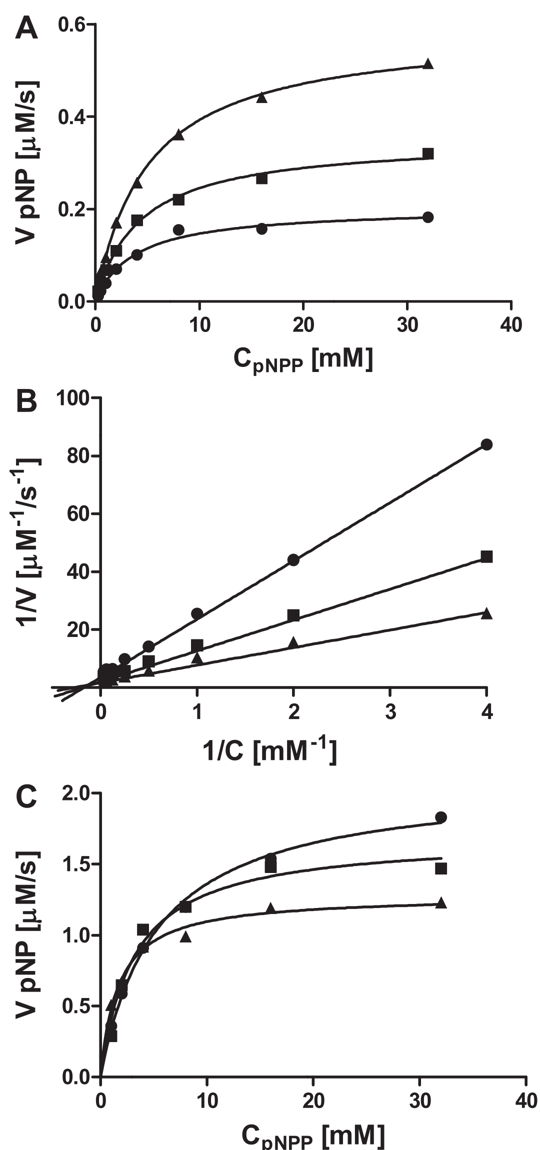

Fig. 2. Recombinant TcPTP1 kinetic analysis and inhibition analysis using BZ3. (A) Non-linear regression analysis of the Michaelis–Menten plot. (B) Lineweaver–Burk plot. Activity was determined using 0.25 μM (circles), 0.5 μM (squares) and 1 μM (triangles) of TcPTP1 and different concentrations of pNPP. (C) TcPTP1 inhibition by BZ3. Circles: 0 μM BZ3; squares: 10 μM BZ3; triangles: 100 μM BZ3.

induce metacyclogenesis *in vitro*, a 6×10^7/mL epimastigote culture was concentrated to 5×10^8 cells/mL and incubated in TAU medium (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 8 mM sodium phosphate buffer, pH 6.0) for 2 h at 28 °C. The parasites were then diluted in TAU3AAG medium (TAU medium supplemented with 50 mM sodium glutamate, 10 mM L-proline, 2 mM sodium aspartate, 10 mM glucose) to a final concentration of 5×10^6 cells/mL [18] and incubated with 100 μM of BZ3, a PTP-1B phosphatase inhibitor (Calbiochem), diluted in DMSO or DMSO (control) for 96 h at 28 °C. Culture supernatants were collected after 96 h and the number of totally differentiated trypomastigotes was determined by fluorescence microscopy after fixation in 4% p-formaldehyde in PBS and staining with 10 μg/mL 4,6-diamidino-2-phenylindole (DAPI).

2.6. Infection assay

Trypomastigotes of the *T. cruzi* Y strain were pre-treated with 100 μM of BZ3 (diluted in DMSO) or DMSO alone for 1 h at 28 °C and then washed to remove completely any traces of BZ3 and DMSO. Thereafter, 6×10^5 of these trypomastigotes were incubated with 2×10^4 L6E9 (ATCC) rat skeletal muscle cells (30:1 parasite to host-cell ratio) for 2 h at 37 °C. The L6 cells were washed with PBS to remove parasites in the medium and the number of infected cells was determined by fluorescence microscopy (after DAPI staining) as described previously [19].

3. Results and discussion

PTPs are important proteins in the cell signaling of eukaryotes. Recently, several PTPs from trypanosomatids have been described [8,10,20]. To obtain more data on the PTP1 from *T. cruzi* (TcPTP1), we have cloned and expressed this protein in *Escherichia coli* using an expression vector with an N-terminal His-Tag. Four hours of induction with relative high amounts of IPTG (1 mM) at temperatures of 30 °C were found to generate maximal levels of soluble protein (shown in Fig. 1A). Higher temperatures and longer induction times did not lead to an improvement of the quantity of expressed TcPTP1 (data not shown).

The fact that PTP1 expresses in soluble form at usual temperatures indicates that this phosphatase, as often the case for protein phosphatases, is not toxic to *E. coli*, and has an acceptable solubility. Indeed several larger scale fermentations (4 L) allowed us to obtain highly purified protein after the affinity step of Ni-affinity purification, as indicated in Fig. 1B. We therefore abstained from subsequent purification steps. The protein was eluted from the Ni-affinity column using about 100 mM imidazol. After dialysis to remove imidazol and salts from the protein solution we were then able to concentrate the protein using microfilters to up to 10 mg/mL. The total yield of protein amounted to 15 mg/L of culture broth.

To biochemically characterize TcPTP1, we then set to establish a Michaelis–Menten steady state assay using the protein tyrosine phosphatase pseudo-substrate pNPP. We employed three concentrations of the protein and eight concentrations of pNPP to fit the Michaelis–Menten equation, as shown in Fig. 2A. Linearization of these curves after Lineweaver–Burk is shown in Fig. 2B, indicating the good fit of the experiment. Averaging values obtained by non-linear regression analysis, we can conclude that the recombinant TcPTP1 is active with a K_m of 4.5 ± 0.3 mM and a k_{cat} of 2.8 ± 0.2 s^{-1}. These values are comparable in magnitude to those obtained by others for human PTP1B [21] and PTP1 from the *T. brucei*, the causing agent of African Sleeping Disease [7].

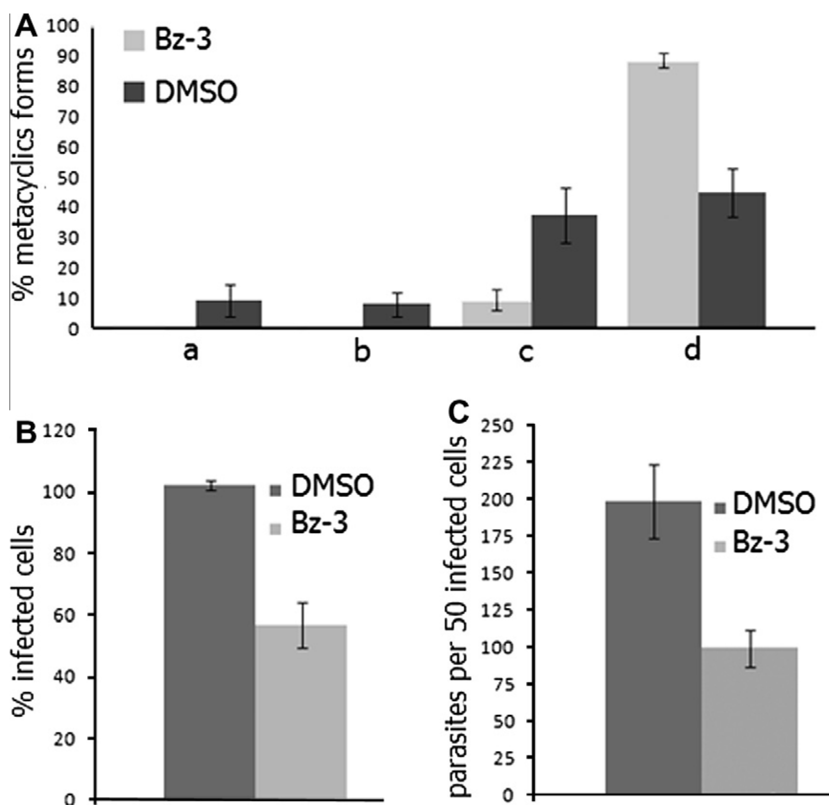

Fig. 3. TcPTP inhibition affects metacyclogenesis and infectivity of *T. cruzi*. (A) Acceleration of the differentiation of *T. cruzi* into the metacyclic form using BZ3. Percentage of parasites after inducting differentiation of epimastigote forms into metacyclic forms, as determined by fluorescence microscopy. (a–c) Shows the percentage of different intermediate metacyclic forms based on their morphology after 96 h, where (d) can be considered the end stage of the metacyclic form. Inhibition by BZ3 of the infectivity of L6 rat skeletal muscle cells by *T. cruzi* trypomastigotes. (B) Percentage of *in vitro* infected cells in the presence of 100 μM BZ3 and DMSO control. (C) Number of parasites internalized per 50 infected cells in the presence of 100 μM BZ3 and DMSO control.

To further characterize TcPTP1, we carried out inhibition studies with the PTP1b inhibitor BZ3. BZ3 (3-(3,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide) is thought to bind and inhibit human PTP1b by an allosteric non-competitive mechanism [22]. Our results indicate that BZ3 also inhibits TcPTP1, in a manner that is compatible with a non-competitive inhibitor, shifting k_{cat} more than K_m (Fig. 2C).

To corroborate the functional importance of this enzyme in *T. cruzi*, we carried out *in vivo* cell culture studies using this inhibitor. *T. cruzi* is known to differentiate from the epimastigote form to the metacyclic form in the gut of its insect host vector. As shown in Fig. 3A, BZ3 accelerates the differentiation of epimastigotes into the metacyclic form. Under control conditions, *T. cruzi* differentiate about 50% to the metacyclic form in our experiment. However, when incubated in the presence of BZ3, about 90% of these cells accumulated in late stages of the transformation, suggesting that TcPTP1 participates in this differentiation process of the parasite. A similar role has been suggested for TbPTP from *T. brucei* [7] and was shown to participate in a glycosome signaling pathway [8]. We can however not exclude that the effects of BZ3 in *T. cruzi* are mediated through other phosphatases of *T. cruzi* like TcPTP2.

The involvement of PTPs in cellular differentiation and physiology in mammalians makes these phosphatases interesting targets for the design of drugs. The classical example for this are the continued efforts of the scientific community and the pharmaceutical industry to develop drugs against human PTP1B [23], whose function can be linked to obesity and diabetes. Another example is the recent development of inhibitors by the means of rational drug design targeted against Shp2, a PTP which is strongly linked with Noonan Syndrome and the most common forms of Juvenile Leukemia [13].

To investigate whether inhibition of *T. cruzi* PTP influences infectivity of this parasite, we carried out a separate cellular infection assay, with a different cellular form of *T. cruzi*, termed trypomastigote. Trypomastigotes are the mayor infective and propagative form of *T. cruzi* after infection of human hosts. In our assay, the trypomastigotes were pre-treated with 100 μM BZ3 for 1 h and, after washing out this inhibitor, incubated with L6 rat skeletal muscle cells. As shown in Fig. 3B and C, infectivity of trypomastigotes pre-treated with BZ3 was significantly lowered by about 50% when compared with the infectivity of untreated trypomastigotes. A report described that other protein tyrosine phosphatase inhibitors affect cell invasion only when incubated with the cells and parasites and propose that dephosphorylation reaction participate in cell invasion [24]. Therefore it is also possible that phosphorylation and dephosphorylation events participate in many events of the parasite–cell interaction.

The sequencing of the genomes of trypanosomatids [25] like *T. cruzi* [26], *T. brucei* [27,28] and *Leishmania major* [29] has led to the detection of several PTPs in these species. The molecular functions as well as the signaling pathways in which these proteins are involved in the trypanosomatids are not well understood, although two of these enzymes have been linked to cellular differentiation processes in *T. brucei* and *L. major* [7,30,31]. The present data provide an important step in obtaining more biochemical, structural and cell biological data for TcPTP1, allowing the establishment of screening assays for the design of inhibitors against this protein. We were able to obtain pure and functional TcPTP1 by recombinant expression in *E. coli*. As proof of principle, we were able to inhibit the activity of this enzyme using a selective protein tyrosine phosphatase inhibitor, demonstrating that the same inhibitor accelerates metacyclogenesis of *T. cruzi*, and inhibits the infectivity of *T. cruzi* trypomastigotes, thus indicating that TcPTP1 is an essential and interesting target for design of inhibitors of this organism.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.038.

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