Protein tyrosine phosphatase *TbPTP1*: a molecular switch controlling life cycle differentiation in trypanosomes

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Differentiation in African trypanosomes (Trypanosoma brucei) entails passage between a mammalian host, where parasites exist as a proliferative slender form or a G0-arrested stumpy form, and the tsetse fly. Stumpy forms arise at the peak of each parasitaemia and are committed to differentiation to procyclic forms that inhabit the tsetse midgut. We have identified a protein tyrosine phosphatase (*TbPTP1*) that inhibits trypanosome differentiation. Consistent with a tyrosine phosphatase, recombinant *TbPTP1* exhibits the anticipated substrate and inhibitor profile, and its activity is impaired by reversible oxidation. *TbPTP1* inactivation in monomorphic bloodstream trypanosomes by RNA interference or pharmacological inhibition triggers spontaneous differentiation to procyclic forms in a subset of committed cells. Consistent with this observation, homogeneous populations of stumpy forms synchronously differentiate to procyclic forms when tyrosine phosphatase activity is inhibited. Our data invoke a new model for trypanosome development in which differentiation to procyclic forms is prevented in the bloodstream by tyrosine dephosphorylation. It may be possible to use PTP1B inhibitors to block trypanosomatid transmission.

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Abbreviations used in this paper: BZ3, 3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethylbenzofuran-6-sulfonic acid (4-(thiazol-2-ylsulfamyl)-phenyl)amide; EGFR, EGF receptor; pNPP, p-nitrophenylphosphate.

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for infection and survival of pathogenic species like Salmonella, Yersinia, or Mycobacterium (Black et al., 2000; Lin et al., 2003; Singh et al., 2003). Kinetoplastid parasites such as Trypanosoma spp. and Leishmania spp. occupy an interesting evolutionary niche, being unicellular organisms and among the most diverged representatives of the eukaryotic world. Although intracellular signaling events have not yet been described in detail for these organisms, it is likely that tyrosine phosphorylation will also play a role in cellular processes as in higher eukaryotes. Supporting this, there is evidence that several proteins are phosphorylated on tyrosine residues in kinetoplastids (Parsons et al., 1991; Cool and Blum, 1993) presumably through the activity of dual-specificity protein kinases, as kinetoplastid genomes do not encode any recognizable tyrosine-specific kinases (Parsons et al., 2005). Tyrosine phosphatase activity also shows marked differences among different life cycle stages in both T. brucei and T. cruzi (Bakalar et al., 1995a).

From the precedent in higher eukaryotes, it is likely that phosphotyrosine phosphatases will be also relevant in the control of cell growth and development in kinetoplastids. Supporting this idea, it was recently reported that the heterologous expression of the human PTP1B gene in Leishmania, together with the inhibition of tyrosine kinases, promoted partial differentiation from promastigote to amastigote forms (Nascimento et al., 2003). Here, we demonstrate that the activity of a T. brucei protein tyrosine phosphatase, TbPTP1, exhibits a pivotal function in parasite differentiation. Biochemical characterization of the enzyme demonstrates that it is a tyrosine-specific phosphatase whose enzymatic activity is regulated by pH and changes in its oxidation state. Importantly, when TbPTP1 activity is inhibited by RNAi or biochemically, differentiation to procyclic forms occurs spontaneously in the absence of any exogenous trigger. This response is restricted to a subset of bloodstream cells, which we propose are those already committed to differentiation. Supporting this, tyrosine phosphatase inhibition in a homogeneous population of stumpy forms triggers synchronous, efficient, and complete differentiation to proliferative procyclic forms. These data reveal that a tyrosine phosphatase activity is a key molecular regulator of the initiation of trypanosome differentiation, providing a potential pharmacological target to restrict parasite transmissibility and virulence.

Results

Identification and expression profile of TbPTP1

To identify molecules implicated in the differentiation competence of bloodstream stumpy forms, we searched the T. brucei genome database for molecules that define G1/G0 arrest in other organisms. This revealed a 595-bp fragment with limited sequence similarity to the protein tyrosine phosphatase PTPROt (Aguiar et al., 1999). PTPROt was first identified in mammalian lymphoid organs and is up-regulated in quiescent B cells. The intact T. brucei gene was then isolated by PCR from cDNA, and the complete gene sequence was determined. This was subsequently verified upon completion of the T. brucei genome project. This gene, which we have named TbPTP1 (T. brucei phosphotyrosine phosphatase 1), is positioned on chromosome 10 (Tb10.70.0070).

Previous evidence that protein phosphatase activities were differentially regulated during the trypanosome life cycle (Bakalar et al., 1995b) prompted us to examine the developmental mRNA expression profile of TbPTP1 by Northern blotting. Total RNA was prepared from monomorphic bloodstream slender forms, bloodstream stumpy forms, and in vitro–cultured procyclic forms. This revealed that the TbPTP1 mRNA was expressed in all stages examined, although up-regulated in stumpy forms (1.5–3-fold), which was somewhat variable between samples (Fig. 1 A). To relate mRNA expression to that of the protein, an anti-peptide antibody was raised against the sequence N-AMKQ-KRFGMVQRLEQ-C from the amino acid sequence at position 265–279 in TbPTP1. When reacted against lysates derived from isogenic monomorphic slender and stumpy forms of T. rhodesiensi EATRO 2340 and procyclic forms of T. brucei Lister 427, approximately equal expression of TbPTP1 protein was detected. TbPTP1 expression was also analyzed during synchronous differentiation of bloodstream stumpy forms to procyclic forms. This revealed no transient changes in the protein expression levels of TbPTP1 during the events of differentiation (Fig. 1 B). Finally, analysis of the subcellular localization of TbPTP1 by differential detergent extraction revealed that TbPTP1 associated predominantly with the cytoskeletal fraction in bloodstream forms (Fig. 1 C). Although no association with any discrete structure (e.g., the flagellum) could be detected (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200605090/DC1), a cytoskeletal association for TbPTP1 matches the distribution of several tyrosine phosphatases characterized in other organisms (Howard et al., 1993; van Ham et al., 2005). This compartmentalization is believed to contribute to their substrate specificity.

Figure 1. TbPTP1 is uniformly expressed during the trypanosome life cycle and localizes with the cytoskeletal fraction. (A) Northern and Western analysis of TbPTP1 levels in slender (Sl), stumpy (St; both T. rhodesiensi EATRO 2340) and procyclic (Pc; T. brucei s427) cells. Loading controls of ethidium bromide–stained total RNA (for the Northern blot) and α-tubulin (for the Western blot) are shown. (B) Protein expression of TbPTP1 during synchronous differentiation to procyclic forms. (C) Cellular distribution of TbPTP1 in stumpy forms. Cytosolic, membrane, nuclear, and cytoskeletal fractions are shown. TbPTP1 associates with the cytoskeletal fraction. Other cellular markers partition as expected (cPGK, cytosolic; BiP, membrane and cytosolic; and α-tubulin, cytoskeletal and cytosolic; not depicted).
The TbPTP1 is a member of a trypanosome-specific PTP family. The 1.8-kb TbPTP1 mRNA contains an ORF of 921 nucleotides encoding a protein of 306 amino acids with a predicted molecular mass of 34 kDa. A predicted PEST sequence region is located between residues 135 and 152 (Fig. 2, underlined). Analysis of the amino acid sequence of TbPTP1 revealed an N-terminal region (43 residues) with no apparent homology to any other protein and a C-terminal region (263 residues) that is homologous to the human protein tyrosine phosphatases PTP1B and PTpro (24 and 23% identity over the catalytic domain of each). A predicted mass of 34 kDa. A predicted PEST sequence region is located between residues 135 and 152 (Fig. 2, underlined). Analysis of the amino acid sequence of TbPTP1 revealed an N-terminal region (43 residues) with no apparent homology to any other protein and a C-terminal region (263 residues) that is homologous to the human protein tyrosine phosphatases PTP1B and PTpro (24 and 23% identity over the catalytic domain of each). An extensive search of different trypanosomatid databases, identified orthologues of TbPTP1 in T. congolense (TcgpPTP1, with 74.1% identity), T. vivax (TvPTP1, with 62.0% identity), and T. cruzi (TcPTP1, with 61.3% identity). The sequence alignment of these orthologues together with human PTP1B is shown in Fig. 2. When sequence similarity is considered, these values rise to 71–80% between TbPTP1 and trypanosomal PTP1s, compared with 38% to human PTP1B. In contrast, Leishmania major has a syntenic gene encoding a predicted tyrosine phosphatase less closely related to TbPTP1 (42% similarity; LmjF36.2180).

Previous sequence analysis of human PTPs has led to the identification of 10 conserved motifs, some of which are important in substrate binding and catalysis (Andersen et al., 2001). The trypanosomal PTP1 subfamily contains all the landmark motifs present in classical tyrosine-specific phosphatases (Fig. 2). These include the phospho-Tyr binding motif (Fig. 2, M1); the WPD loop (M8), which contains the catalytic aspartic acid (the general acid in catalysis); the catalytic P-loop or PTP signature motif (V/I)HCSAGXGR(T/S)(M9); and the Q-loop (M10), which is part of the active site in classic PTPs. Motifs 3–7 (M3–M7) are also present in trypanosomal PTP1s, with a high percentage of conservation, consistent with their role as structural motifs located in the core of the PTP catalytic domain.

In total, 9 of the 10 well-conserved PTP motifs identified in the mammalian enzymes are present in all the examined trypanosomal PTP1s, with only the less-conserved motif 2 missing. Instead of motif 2, a trypanosome-specific motif, T1, replaces this structural motif.

Other distinct motifs were also identified in the trypanosomal PTP1s, generating a total of four trypanosome-specific motifs in the catalytic region (Fig. 2, T1–T4). These are as follows: T1 at position 55–63, “LANEXTITYP”; T2 at position 165–167, “EVD”; T3 at position 239–244, “LIQAYA”; and T4 at position 290–295, “RLGV (D/S) (I/V).” In addition, two other motifs have been identified in the precatalytic (Pc) region: PcT1 “R (M/L)QREFXQGQL” at position 20–29 and PcT2 “ENPRX1(D/N)FTTSL” at position 32–43. These precatalytic motifs are well conserved in all members of the T. brucei clade (T. brucei, T. congolense, and T. vivax) and less conserved in T. cruzi. A BLAST search failed to identify any of these motifs in other proteins, indicating that they are unique to the trypanosomal PTP1 family. We hypothesize that these trypanosome-specific motifs may be relevant to regulation of TbPTP1 or molecular recognition of other cellular targets.

The activity of TbPTP1 is regulated by reversible oxidation in vitro. Having identified TbPTP1 as a putative tyrosine phosphatase, it was important to characterize its enzymatic activity profile and substrate specificity. For this, recombinant protein was produced for the wild-type protein and for two TbPTP1 mutants, one for the putative catalytic cysteine, Cys 229 (in the P-loop) and one for the catalytic Asp 199 (in the WPD loop). The catalytic mutants of TbPTP1 were generated by mutating Cys 229 to serine (C229S) and Asp 199 to alanine (D199A). The wild-type and mutant enzymes were expressed as His-tagged fusions and purified to >95% homogeneity. After removal of the His tag by enterokinase digestion, multigene

Figure 2. Alignment of TbPTP1 with orthologous trypanosome PTP1s and the human PTP1B. The deduced amino acid sequences encoding TbPTP1 of T. brucei (Tb10.070.0007), T. congolense (Tcog1301101.p1k), T. vivax (Tv1180604.p1k), and T. cruzi (Tc00.104703510187.234), and the human PTP1B (P18031) were aligned using Clustal X (Thompson et al., 1997). The classical PTP motifs are indicated above the sequences in white boxes (M1–M10). The trypanosome-specific motifs are boxed in gray. PcT1 and PcT2 are the catalytic motifs, and the T1–T4 motifs are conserved in the catalytic region. The predicted PEST sequence is underlined.
light scattering analysis of the enzyme showed that TbPTP1 behaves as a monomeric protein in solution, with a determined mass of 35,140 D.

The phosphatase activities of the purified recombinant wild-type, D199A, and C229S enzymes were assayed using various concentrations of p-nitrophenylphosphate (pNPP), a widely used substrate of tyrosine phosphatases. The pH profile analysis of pNPP dephosphorylation demonstrated that the wild-type enzyme has the highest specific activity at pH 6.0 (Fig. 3 A), in agreement with the fact that PTPs in general have optimal enzymatic activities at low pH values. The mutant enzyme D199A showed low activity (5–8% of the wild type), and the C229S mutant enzyme was totally inactive under any assay condition and substrate concentration tested (unpublished data). This confirms our predictions from the sequence analysis and demonstrates that both residues, Cys 229 and Asp 199, are essential in the mechanism of catalysis of TbPTP1, matching their orthologues in mammalian PTPs. Steady-state kinetic analysis of the wild-type enzyme yielded a $V_{\text{max}}$ value of 0.12 mM min$^{-1}$ and a $k_{\text{cat}}/K_m$ of 3.57 mM$^{-1}$ s$^{-1}$ for the dephosphorylation of pNPP. Addition of 2 mM of DTT had a marked effect on the catalytic rate, increasing the $V_{\text{max}}$ by threefold and the $k_{\text{cat}}/K_m$ by 14-fold (Fig. 3 B and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200605090/DC1), consistent with previous observations suggesting that the catalytic cysteine needs to be in a reduced state for efficient nucleophilic attack of the substrate (Denu and Tanner, 1998). To investigate whether redox events (Denu and Tanner, 1998; Salmeen et al., 2003; van Montfort et al., 2003) could influence the enzymatic activity of TbPTP1, we performed a time course activity assay using increasing amounts of the oxidizing agent, hydrogen peroxide (H$_2$O$_2$). Fig. 3 C shows that after 10 min of incubation, the addition of 0.25 mM H$_2$O$_2$ completely inactivated TbPTP1 and 0.025 and 0.1 mM H$_2$O$_2$ reduced the enzyme activity by 37 and 58%, respectively. Importantly, the enzymatic activity of the inactive TbPTP1 toward pNPP was completely restored after 5 min of incubation with 10 mM DTT. These experiments indicate that TbPTP1 is sensitive to reversible redox regulation, consistent with several characterized mammalian PTPs (Denu and Tanner, 1998).

**TbPTP1 is a tyrosine-specific protein phosphatase**

To test the specific activity of TbPTP1, a full kinetic characterization of the wild-type enzyme was undertaken using a range of phosphorylated substrates. These included tyrosine-, serine-, and threonine-phosphorylated peptides; phospho- amino acids; nucleotides; phospholipids; and inorganic phosphorylated compounds (Fig. 4 A and Table S1). Most important, this analysis showed that TbPTP1 favored tyrosine-phosphorylated substrates in the dephosphorylation assays while showing 10–40-fold less activity against the Ser/Thr-phosphorylated substrates. In contrast, TbPTP1 exhibited little or no activity toward the phospholipids, nucleotides, or inorganic phosphocompounds tested, suggesting that the purified enzyme does not exhibit either lipid phosphatase or phosphoesterase activity. Analysis of the saturation kinetics of TbPTP1 was also performed using the Tyr-phosphorylated EGF receptor peptide (pEGFR), the Insulin receptor peptide (pInsulin), phospho-Tyr (pTyr), and phospho-Ser (pSer). The results obtained showed that TbPTP1 dephosphorylates the pEGFR peptide with the highest $V_{\text{max}}$, 1.5–2 times higher than for pInsulin peptide and pTyr, and six times higher than for pSer. As activity toward nucleotides, phospholipids, and inorganic phosphocompounds was considerably lower or nonmeasurable, no further kinetic analysis was considered relevant for these compounds.

In a final series of biochemical experiments, the inhibitor profile of TbPTP1 was investigated. The phosphatase activity of TbPTP1 was assayed using pNPP or pEGFR peptide in the presence of different inhibitors. The PTP-specific inhibitor
sodium orthovanadate impaired the catalytic activity of TbPTP1 in a concentration-dependent manner (Fig. 4 B). Dephosphorylation of both pNPP and of the pEGFR peptide was reduced by 90% by 1 mM sodium orthovanadate, and the activity was completely abolished by 10 mM of this PTP inhibitor. In contrast, the Ser/Thr phosphatase inhibitor sodium fluoride had no measurable effect on the activity of TbPTP1. Concentrations and names of the inhibitors (OA, okadaic acid; Tetram, tetramisole) are indicated on the x axis. The fraction (%) of residual activities in the presence or in the absence of inhibitors is shown on the y axis. The filled columns show activity measured using pNPP as substrate, and the empty columns show the phosphatase assays performed using pEGFR as substrate. All data points represent the mean values of triplicate determinations, and error bars (± SEM) are indicated.

Figure 4. TbPTP1 shows a tyrosine-specific protein phosphatase activity and inhibitor profile. (A) Various phosphosubstrates (2 mM final concentration) were assayed using a malachite green detection system. The tyrosine-phosphorylated EGFR (DADEpYLIPQQG) and insulin receptor (TRDIpYETDYYRK) were dephosphorylated with the highest rate followed by phosphoTyr amino acid. Other phosphosubstrates proved to be poor substrates for TbPTP1. (B) Effect of various inhibitors on the activity of TbPTP1. Concentrations and names of the inhibitors (OA, okadaic acid; Tetram, tetramisole) are indicated on the x axis. The fraction (%) of residual activities in the presence or in the absence of inhibitors is shown on the y axis. The filled columns show activity measured using pNPP as substrate, and the empty columns show the phosphatase assays performed using pEGFR as substrate. All data points represent the mean values of triplicate determinations, and error bars (± SEM) are indicated.

We successfully generated several viable cell lines, although in only one case was effective ablation of TbPTP1 mRNA and loss of protein observed, and this generated a subtle growth phenotype (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200605090/DC1). During phenotypic characterization of this cell line, we observed that a small proportion (2–12%) of cells spontaneously differentiated to procyclic forms despite maintenance of the cells in bloodstream form culture conditions (HMI-9 culture medium at 37°C). This phenotype manifested itself as the presence of cells in the population that stained strongly with antibodies specific for both EP and GPEET (procyclin) (Roditi and Clayton, 1999). Significantly, this did not represent loss of procyclin gene expression control alone (as can be observed at a frequency of up to 0.1% in wild-type bloodstream populations); analysis of the procyclin-stained cells indicated that they also exhibited characteristic procyclic-form morphology, with their mitochondrial genome (kinetoplast) being positioned away from the cell posterior (Matthews et al., 1995; Fig. 5 B). They also expressed the procyclic stage–specific cytoskeletal antigen CAP5.5 (Hertz-Fowler et al., 2001; unpublished data).

Unfortunately, the differentiation phenotype was rapidly unstable and thus difficult to maintain in culture, with the proportion of differentiated cells decreasing in successive passages, even in the absence of induction.

The instability of the phenotype observed with TbPTP1 RNAi prompted a search for alternative approaches to target the activity of this enzyme. Recently, a series of highly specific sulphonamido-benzabromarone allosteric inhibitors of PTP1B have been reported (Wiesmann et al., 2004), of which 3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide (BZ3) exhibits good
cell permeability. This compound prevents closure of the PTP1B WPD loop, thereby preventing dephosphorylation of substrates.

To investigate whether this would provide a useful reagent for functional analysis of TbPTP1, we tested the inhibition profile and specificity of BZ3 against TbPTP1 and other PTPs. We found that 10 μM of BZ3 reduced the activity of TbPTP1 at least 50%, consistent with its activity against human PTP1B (IC50 = 8 μM; Wiesmann et al., 2004), whereas a dual-specificity phosphatase and the human low molecular weight phosphatase HCPTPB, which lacks the WPD loop, showed no inhibition at this concentration (Fig. 6).

Having verified the specificity of BZ3 for TbPTP1, we exposed monomorphic bloodstream forms in culture to a titration of BZ3 ranging from 50 to 150 μM, which was in the same range as the concentration effective against mammalian PTP1B in CHO cells in culture (250 μM; Wiesmann et al., 2004). Strikingly, this also resulted in spontaneous differentiation of a subset of the parasites in response to the inhibitor, with 150 μM BZ3 stimulating procyclin expression in the bloodstream population at a frequency of 9.4% (Table I). Moreover, the phenotype of the cells was consistent with that observed in the RNAi line; i.e., the procyclin expressers in the population also exhibited kinetoplast repositioning, indicative of morphological differentiation (unpublished data). In contrast, exposure of monomorphic bloodstream forms to a non–cell-permeable PTP inhibitor, sodium orthovanadate (at 1 mM), did not result in detectable differentiation, nor did exposure to 1 mM NaF, a Ser/Thr phosphatase inhibitor (Table I). Thus, TbPTP1 inhibition promotes spontaneous differentiation to procyclic forms in the absence of exogenous trigger in a subset of bloodstream forms in culture.

To verify that TbPTP1 was a target of BZ3 in vivo and that this was linked to the differentiation phenotype observed, transgenic bloodstream cell lines were generated which ectopically expressed wild type or a D199A mutant of TbPTP1, which binds but not release substrates (Tonks, 2003), acting as a dominant-negative mutation (Fig. 7). When exposed to 150 μM BZ3, cells expressing the D199A mutant reproducibly exhibited significantly enhanced differentiation when compared with the same cell line in the absence of tetracycline induction (18.1% in the induced population versus 11.6% in the uninduced population; Tukey post hoc comparison, P = 0.049). In contrast, no enhanced differentiation was observed in cells that ectopically expressed the wild-type TbPTP1 (9% in the induced population versus 10.3% in the uninduced population; P = 0.86). This demonstrates that expression of the D199A mutant of TbPTP1 increases differentiation in response to BZ3. This provides further evidence that TbPTP1 is a target of BZ3 in vivo and that this is linked to the observed differentiation phenotype.

**Table I.** Percentage of EP procyclin–positive cells in monomorphic cultures treated with phosphatase inhibitors

| Treatment | EP procyclin–positive cells (%) |
|-----------|---------------------------------|
| Untreated monomorphic cells | 0.3 |
| + DMSO | 0 |
| + 50 μM BZ3 | 1.6 |
| + 100 μM BZ3 | 5.2 |
| + 150 μM BZ3 | 9.4 |
| + 1 mM NaOVanadate | 0.6 |
| + 1 mM NaF | 0.2 |

Cell samples were processed after 18 h under each condition.

**Figure 6.** BZ3 (0, 2, or 10 μM) inhibition of recombinant TbPTP1, human low molecular weight phosphatase (HCPTPB), and a mammalian dual-specificity phosphatase. In each case, pNPP was used as substrate. Error bars indicate SEM.

**Figure 7.** BZ3 induced differentiation in bloodstream forms expressing wild-type TbPTP1-Ty or the D199A TbPTP1 mutant. For each, transgene expression was induced (+ Tet) or not (− Tet) for 3 d, with cells then being exposed to 1.50 μM BZ3. Expression of EP procyclin was assayed in 500 cells after 24 h. Induction of each ectopically expressed protein was verified by Western blotting using an antibody (BB2) directed against the Ty-1 epitope tag incorporated into each protein. The results of three independent experiments are shown, with error bars indicating the SEM. There was no difference in growth kinetics between each line before BZ3 addition (not depicted).

**BZ3 stimulates synchronous differentiation of stumpy forms**

We wished to investigate why only a subset of monomorphic bloodstream trypanosomes underwent spontaneous differentiation...
to procyclic forms when TbPTP1 was inhibited. Monomorphic bloodstream forms are so named because they have lost the capacity to generate morphologically stumpy forms through prolonged laboratory passage. However, they do retain the capacity to differentiate to procyclic forms when stimulated with cis aconitate, although this is asynchronous in the population and of variable efficiency. We previously proposed that this asynchrony arises from the requirement for individual cells to undergo commitment to differentiation (Matthews and Gull, 1994; Tasker et al., 2000). These committed cells remain slender in morphology but are functionally equivalent to stumpy forms in terms of their ability to differentiate, a condition we have termed stumpy* (Tasker et al., 2000; Matthews et al., 2004). We hypothesized that the small proportion of monomorphic cells that differentiated in response to BZ3 or TbPTP1 RNAi were these stumpy* forms, which was supported by the increased differentiation of these RNAi lines when incubated with a cAMP analogue reported to promote stumpy formation (Fig. S2 C). To further evaluate this, we investigated the response to BZ3 of homogenous populations of stumpy forms, which are uniformly and irreversibly committed to differentiation to procyclic forms. Our prediction was that these cells would show a highly efficient differentiation to procyclic forms in response to BZ3 when compared with cultured monomorphic lines.

Initially, we assayed BZ3-induced differentiation in pleomorphic slender cells derived from a rodent infection. This resulted in an ~7% differentiation after 24 h, consistent with the response of cultured monomorphic cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200605090/DC1). Thereafter, trypanosome populations highly enriched for stumpy forms were harvested from a mouse infection, and these cells were exposed either to 150 μM BZ3, 6 mM cis aconitate, or 0.3% vol/vol DMSO (used to solubilize BZ3). In all cases, the cells were then maintained in bloodstream-form culture conditions (37°C in HMI-9) over a period of 24 h, conditions that are compatible with both efficient differentiation of cis aconitate–stimulated cells (Matthews and Gull, 1997) and maintenance of the viability of undifferentiated stumpy forms. Samples were harvested at various time points and analyzed for the expression of EP procyclin by immunofluorescence and for morphological differentiation by quantitative analysis of the kinetoplast–posterior dimension in each cell population. Figs. 8 and 9 demonstrate the remarkable result of this analysis: stumpy populations differentiated to procyclic forms synchronously and efficiently when exposed to BZ3, with the kinetics of this being equivalent to cells exposed to cis aconitate (Matthews and Gull, 1994). Thus, by 6 h in 150 μM BZ3, >70% of cells were EP procyclin positive (Fig. 8 A), and by 24 h the kinetoplast–posterior dimension had increased from ~1 μm (equivalent to that seen in bloodstream forms) to ~5 μm (equivalent to that in procyclic forms; Matthews et al., 1995; Fig. 8 B). In contrast, cells exposed to DMSO alone remained as bloodstream stumpy forms and showed no evidence of differentiation to procyclic forms (Fig. 9 A). Moreover, by 24 h, differentiated cells in the 150 μM BZ3–treated population were undergoing cell division as differentiated procyclic forms and had induced expression of the procyclic stage–specific cytoskeletal protein CAP5.5 (Matthews and Gull, 1994; Hertz-Fowler et al., 2001; Fig. 9, B and C). We conclude that inhibition of TbPTP1 stimulates committed bloodstream-form trypanosomes to differentiate to procyclic forms in the absence of any previously described trigger for this process.

**Discussion**

In this study, we report the identification, biochemical characterization, and functional analysis of a novel *T. brucei* protein tyrosine phosphatase, TbPTP1, implicated in life cycle differentiation control in *T. brucei*. TbPTP1 was originally identified in a search for molecular markers of the nondividing bloodstream form of *T. brucei*, by analogy to other molecules associated with G0 arrest in eukaryotic cells. Bioinformatics analysis of the TbPTP1 sequence and further searches in other kinetoplastid genomes revealed the existence of orthologue protein tyrosine phosphatases in the other trypanosomatids *T. vivax*, *T. congolense*, and *T. cruzi*. All of them contain the well-defined motifs conserved in classical PTPs, necessary for catalysis and substrate binding. In addition, distinct motifs are conserved in the
trypanosomal subfamily of phosphatases, both in the catalytic region and in the N-terminal region, upstream of the catalytic domain. We suggest that these *Trypanosoma* spp.–specific motifs could be important in functional regulation of *TbPTP1*.

When expressed as a recombinant protein, *TbPTP1* preferentially dephosphorylated phosphotyrosine substrates and was inhibited in vitro by sodium orthovanadate, a known tyrosine-specific phosphatase inhibitor. Moreover, the enzyme was regulated by reversible oxidation and mutation of the predicted essential catalytic residues C229 and D199 resulted in proteins with either severely impaired enzymatic activity (D199A) or total inactivity (C229S). Together, these results confirm the assignment of *TbPTP1* as a phosphotyrosine-specific phosphatase. This contrasts with previously reported phosphatases in *T. brucei*, for which our sequence analysis failed to find any of the conserved PTP-specific motifs or any homology to the classical PTPs (Bakalar et al., 2000). Thus, *TbPTP1* is to our knowledge the first report of a cloned gene encoding a bona fide nonreceptor tyrosine-specific phosphatase in these organisms.

The biological function of *TbPTP1* was investigated by two experimental approaches: gene-specific RNAi and using a cell-permeable PTP1B-specific inhibitor in vivo. The first approach implicated *TbPTP1* in life cycle regulation, with spontaneous differentiation being observed in a subset of cultured bloodstream-form cells. However, this phenotype was unstable, and the proportion of differentiated cells was reduced to wild-type levels with continued culture of the parasites whether cells were induced or not. This is not surprising, as transfection procedures and continued passage at 37°C in bloodstream-specific culture media would quickly select against populations that generate procyclic forms. Moreover, other functions of *TbPTP1* in proliferative bloodstream forms cannot be excluded. Therefore, we also analyzed the effect of a recently characterized cell-permeable allosteric inhibitor of PTP1B, a benzobromarone derivative (Wiesmann et al., 2004). This inhibitor prevents the closure of the WPD loop of PTPs, therefore keeping the enzyme in a catalytically inactive form. We demonstrated that this inhibitor was effective against *TbPTP1*, but not against the human low molecular weight PTP (LMWPTP) or a dual-specificity phosphatase. Analysis of the phosphatase complement of the complete *T. brucei* genome identified only two putative proteins with an intact WPD loop motif and predicted tyrosine phosphatase activity. One of these is *TbPTP1*, and we have confirmed its BZ3 sensitivity here. The second enzyme, which we term *TbPTP2* (Tb11.01.5450), is not likely to contribute to the observed differentiation phenotype, as RNAi against this enzyme does not elicit differentiation of bloodstream forms (unpublished data). Although we cannot exclude other potential uncharacterized targets in trypanosomes, the compatible phenotypes resulting from genetic and pharmacological inhibition of *TbPTP1* implicate this enzyme as being responsible for the differentiation phenotype. Moreover, specificity of the response was further supported by the enhanced differentiation observed in BZ3-treated cells expressing the dominant-negative D199A mutant of *TbPTP1*.

Only a small subset of bloodstream-form cells in asynchronous in vitro cultures were stimulated to differentiate to procyclic forms by BZ3. However, the most striking observation was the effect of this inhibitor on uniform populations of stumpy forms. Here, treatment with the PTP1B inhibitor resulted in efficient differentiation to procyclic forms. We observed that stage-regulated protein expression (EP procyclin; CAP5.5), kinetoplast repositioning, and cell cycle progression all occurred with equivalent efficiency and on the same time scale as cells treated with cis aconitate, an established trigger for trypanosome differentiation. We interpret the differentiation of the small proportion of cells in bloodstream cultures as the response of the subset of cells in this population, which have entered division arrest or committed to other early events in stumpy formation, before morphological transformation. We previously referred to these cells as stumpy* forms (Tasker et al., 2000).

In Fig. 10, we present a model for the role of *TbPTP1* in the control of bloodstream to procyclic differentiation. In this model, bloodstream parasites commit to stumpy formation and become competent (stumpy*) to differentiate to procyclic forms. However, they are held arrested in this state in the bloodstream by the action of *TbPTP1*. Then, upon uptake by the tsetse fly, this inhibition is removed and the parasites progress unhindered to procyclic forms, progressing through a highly programmed developmental pathway in which cellular events occur on a predetermined pathway and time scale. This model has two important implications. First, it supports the concept that proliferative slender cells are not competent to differentiate to procyclic forms unless they commit to the early events of stumpy formation, in particular, cell cycle arrest. Also, it
serves as an example of the process that is involved in transmissibility.

This model does not predict what is the natural signal that triggers the inactivation of TbPTP1, although one possibility is that the different conditions to which the trypanosome is exposed upon entering the fly. For example, pH fluctuations have been observed in the tsetse digestive tract from pH 9.0 to pH 10.2 (Linner et al., 2003), at which the activity of TbPTP1 is considerably lower. Potentially combined with other specific signals, for example, changes in redox conditions upon tsetse uptake, this would then inactivate TbPTP1 and license the trypanosome for cell cycle reentry and differentiation into a proliferative procyclic form.

The proposed role of TbPTP1 in preventing inappropriate differentiation of stumpy forms in the mammalian bloodstream is essential for transmissibility of the parasite. If not strictly controlled, the progression to procyclic forms would result in rapid death of the stumpy population in the bloodstream because of either activation of complement by the alternative pathway (killing cells that have lost the variant surface glycoprotein) or generation of antibodies to invariant procyclic surface antigens. This makes TbPTP1 a key component of the regulation of the trypanosome life cycle and hence a potentially important pharmacological target for controlling trypanosome transmission, for example, in epidemic foci involving intensive human–human or human–livestock transmission. If coupled with factors to promote stumpy formation, targeting TbPTP1 may also help limit parasite virulence and promote trypanosome clearance. There is intense interest in the development of PTP1B inhibitors in the pharmaceutical industry because of the importance of these enzymes in diabetes and obesity (Ukkola and Santaniemi, 2002; Zhang and Lee, 2003), and we are currently investigating the opportunity for piggyback strategies to target kinetoplastid PTPs. Significantly, the presence of trypanosome-specific motifs in this enzyme offers the potential for developing inhibitors with specificity for the parasite enzyme with respect to their mammalian counterparts.

**Materials and methods**

**Materials**

Phosphosubstrates and other chemicals were purchased from Sigma-Aldrich. The Threonine (KRpTIRR) and Serine (RRApSVA) phosphopeptides were obtained from Upstate. The PTP1B inhibitor BZ3 was purchased from Calbiochem.

**Parasite growth and differentiation**

Parasite lines used were T.b. rhodesiense EATRO 2340 GUP2965 (morphomorphic slender forms) or, for stumpy generation, the isogenic pleomorphic line GUP2962 (Matthews and Gull, 1994). For RNAi analyses, T.b. brucei Lister 427 single-marker cells were used (a gift from G. Cross, Rockefeller University, New York, NY; Wirtz et al., 1999), whereas established procyclic forms were T.b. brucei Lister 427.

For BZ3 inhibition assays, cells in HMI-9 medium (Hirumi and Hirumi, 1989) at 37°C were exposed to DMSO, PTP1B inhibitor BZ3, or 6 mM cis aconitate. Samples were assayed by immunofluorescence at room temperature using antibody to EP procyclin (diluted 1:500 in PBS; Cedar Lane Laboratories), antibody to GPEET procyclin (diluted 1:200; a gift from I. Roditi, University of Bern, Bern, Switzerland), or the procyclin form cytoskeletal protein CAP5.5 (undiluted hybridoma supernatant; a gift from K. Gull, University of Oxford, Oxford, England; Hertz-Fowler et al., 2001). Secondary antibodies were goat anti-mouse (EP procyclin; CAP5.5) or –rabbit (GPEET procyclin) conjugated to either FITC (1:50; Sigma-Aldrich) or Alexa 488 (1:200; Invitrogen). Slides were stained with 1 μg/ml DAPI and mounted in MOWIOL (Harlow Chemical Co.). Morphometric measurements of kinetoplast repositioning used Scion Image 1.62. Images were captured using a Cool charged-coupled device camera attached to an Axioscope 2 (Carl Zeiss Microlmaging, Inc.) using either Plan Neofluar 100× (1.25 NA) or Plan Neofluor 100× (1.30 NA) phase-contrast objectives. Images were processed using Photoshop CS (Adobe).

**DNA cloning**

A 593-bp fragment related to PTPRO (Aguirue et al., 1999) formed part of the sheared T. brucei strain TREU 927/4 genomic DNA clone 18E19 (available from GenBank/EMBL/DDBJ) under accession no. AG649023). Oligonucleotides were designed to the 5′ end of the ΔPTP A, 5′-CATCAACCTCGTACCGACG-3′ and 3′ end of the ΔPTP B, 5′-CAAGCCATACAAATGTGGC-3′ ends of the gene fragment, and these were used in independent amplifications with primers specific for the truncated leader sequence or polyA tail to amplify the gene in two overlapping halves from cDNA. The sequence of the full gene was determined after completion of the trypanosome genome sequence.

**Northern and Western blotting**

RNA preparation and Northern blotting was performed as described by Tasker et al. (2000) using a digoxigenin-labeled ΔPTP1-specific riboprobe (Roche). Stumpy cell fractionation used the Qproteome kit (QIAGEN) with each fraction being probed with antibodies to α-tubulin (cytoskeletal fraction; a gift from K. Gull), cytosolic PGK (cytosolic; a gift from P. Michels, Université Catholique de Louvain, Brussels, Belgium), and BIP (membrane and organelles; a gift from J. Bangs, University of Wisconsin, Madison, WI) as controls. For detection of ΔPTP1, an anti-peptide [NH2-AMIKGWGFMGVRQQC-OH] antibody was raised in rabbits and affinity purified against the immunogen (Eurogentec). Preimmune serum detected no trypanosome protein. Western blotting was performed according to Tasker et al. (2000).
Phosphatase assays using phosphosubstrates

Dephosphorylation of phosphosubstrates was detected by measuring the release of inorganic phosphate with the malachite green detection system according to the manufacturer’s protocol (Protein tyrosine phosphatase assay kit, Sigma-Aldrich). Reaction mixtures (50 μl) contained 5–10 μg of purified TbPTP1 with concentrations from 0.01 to 0.2 mM of phosphopeptides in 50 mM HEPES and 150 mM NaCl, pH 7.0. Reactions were incubated at 37°C for 15–30 min and quenched by adding of 50 μl of malachite green reagent. After 15 min of further incubation at room temperature, the absorbance of the samples were measured at 620 nm in a microplate reader (OpriSyr M; Dynex Technologies), with released inorganic phosphate being determined using a phosphate standard curve. The specific activity is defined as moles of inorganic phosphate released in a minute per milligram of protein. Kinetic constants K_m and V_max were then linearized by NotI digestion and transfected as previously described.

Specific activity was calculated using the Lineaweaver-Burke plot of the reciprocal initial velocity versus the reciprocal concentration of substrates. See the supplemental text (available at http://www.jcb.org/cgi/content/full/jcb.200605090/DC1) for information about protein expression constructs and recombinant protein purification.

Phosphatase assays using pNPP

Enzyme activity was measured by monitoring TbPTP1 (5–20 μg) catalyzed hydrolysis of pNPP to pnitrophenol (Zhang and Van Etten, 1990). A final concentration of 20 mM pNPP was present in the assay. The pNPP assay buffer was 50 mM Tris, 50 mM bisTris, and 100 mM Na acetate, pH 5–7.5. Each reaction (400 μl) was performed in triplicate, being incubated at 37°C for 15 min and quenched by adding 500 μl of 1 M NaOH. The concentration of released pnitrophenol, determined at 405 nm, was converted to millimolar units using a millimolar extinction coefficient of 18.0 mM cm⁻¹.

Reversible inactivation of TbPTP1 by H₂O₂

The assays (120 μl) contained 60 μg of TbPTP1 and 0–0.25 mM H₂O₂ in 50 mM HEPES, pH 7.0, and 150 mM NaCl. Reactions, initiated by addition of H₂O₂, were incubated at room temperature. After 15 min, 10 mM DTT was added. 20-μl samples removed at 0, 5, 10, 15, 20, 30, and 40 min were assayed for residual activity using pNPP as substrate.

Online supplemental material

Table S1 shows kinetic constants for the hydrolysis of phosphosubstrates by TbPTP1. Fig. S1 shows immunofluorescence localization of ectopically expressed TbPTP1. Fig. S2 shows growth kinetics, RNA, and protein expression of the TbPTP1 RNAi line and the response of the TbPTP RNAi line to pCPCAMP. Fig. S3 shows B23 response of pleomorphic slender cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200605090/DC1.

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