

Type 3 peptide deformylases are required for oxidative phosphorylation in *Trypanosoma brucei*

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Summary

Peptide deformylase (PDF) catalyses the removal of the formyl group from the first methionine of nascent proteins. Type 1 PDFs are found in bacteria and have orthologues in most eukaryotes. Type 2 PDFs are restricted to bacteria. Type 3 enzymes are found in Archaea and trypanosomatids and have not been studied experimentally yet. Thus, TbPDF1 and TbPDF2, the two PDF orthologues of the parasitic protozoa *Trypanosoma brucei*, are of type 3. An experimental analysis of these enzymes shows that both are mitochondrially localized, but that only TbPDF1 is essential for normal growth. Recombinant TbPDF1 exhibits PDF activity with a substrate specificity identical to that of bacterial enzymes. Consistent with these results, TbPDF1 is required for oxidative but not for mitochondrial substrate-level phosphorylation. Ablation of TbPDF2, in contrast, does neither affect growth on standard medium nor oxidative phosphorylation. However, a reduced level of TbPDF2 slows down growth in a medium that selects for highly efficient oxidative phosphorylation. Furthermore, combined ablation of TbPDF1 and TbPDF2 results in an earlier growth arrest than is observed by downregulation of TbPDF1 alone. These results suggest that TbPDF2 is functionally linked to TbPDF1, and that it can influence the efficiency of oxidative phosphorylation.

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Introduction

Bacterial and organellar initiator tRNAs^{Met} are only functional when the methionine they carry is formylated. Addition of the formyl group to the charged initiator tRNA^{Met} is catalysed by the methionyl-tRNA^{Met} formyltransferase (MTF). During translation initiation, the formylated initiator tRNA^{Met} interacts with the bacterial-type initiation factor 2, and the resulting complex binds to the ribosome in a GTP-dependent way (Mayer *et al.*, 2001). Thus, newly synthesized proteins emerging from ribosomes of bacteria and organelles carry formyl-methionines at their N-termini. In many cases, this formyl-methionine needs to be removed for the protein to be functional (Giglione and Meinzel, 2001a; Giglione *et al.*, 2004). However, aminopeptidases, which cleave off N-terminal methionines, can only process methionines that are not formylated. Therefore, a peptide deformylase (PDF) is needed which removes the formyl group (Giglione and Meinzel, 2001a; Giglione *et al.*, 2004).

For many years it was thought that PDFs are specific for bacteria, and they were therefore considered to be excellent targets for antibacterial drugs. Indeed, several PDF inhibitors are being tested in clinical trials (Boularot *et al.*, 2004; Leeds and Dean, 2006). However, more recent studies have shown that PDF homologues are also encoded in essentially all eukaryotic and in some archaeal genomes. The few eukaryotic PDFs that have been studied in more detail are essential proteins (Giglione *et al.*, 2003; Lee *et al.*, 2004) that are targeted to chloroplasts and to mitochondria (Lee *et al.*, 2004; Nguyen *et al.*, 2003; Serero *et al.*, 2003).

Trypanosoma brucei is a parasitic protozoa that occurs in sub-Saharan Africa. It has a complex life cycle and is transmitted between mammalian hosts through a tsetse fly vector. *T. brucei* causes human sleeping sickness and various diseases in other animals. If left untreated, sleeping sickness is invariably fatal. However, treatment of the disease is still in a very unsatisfactory state, and the only effective drug against the late stage of the disease, which is in use for more than 50 years, causes death in 3–10% of the patients (Lüscher *et al.*, 2007). *T. brucei* is related to *Trypanosoma cruzi*, the causative agent of Chagas disease in Latin America, as well as *Leishmania* spp. that occur in much of the tropical and subtropical world and that cause the different forms of leishmaniasis.

As in other eukaryotes, mitochondrial translation in the parasitic protozoa *T. brucei* is of the bacterial-type (Schneider, 2001). Consistent with this, it was shown that mitochondrial translation initiation in *T. brucei* requires a formylated tRNA^{Met} (Tan *et al.*, 2002). However, due to the complete lack of mitochondrial tRNA genes (Schneider and Marechal-Drouard, 2000; Bhattacharyya and Adhya, 2004), it is not a bacterial-type initiator tRNA^{Met} that becomes formylated, but the elongator tRNA^{Met} that is imported from the cytosol. The formylation is catalysed by an MTF that, unlike its counterparts in other organisms, recognizes the imported elongator tRNA^{Met}. Thus, we find the surprising situation in *T. brucei* mitochondria that the elongator tRNA^{Met} is functional in translation initiation provided that it is formylated (Martin, 2002). This highlights how crucial tRNA formylation is in mitochondria. In agreement with these findings, it was shown that the main determinant recognized by the mitochondrial initiation factor 2 of *T. brucei* is the formylated methionine (Charrière *et al.*, 2005). It is therefore clear that newly synthesized mitochondrially encoded proteins of *T. brucei* carry an N-terminal formyl-methionine.

Mitochondrial translation in *T. brucei* has proven to be difficult to study. Thus, except for two mitochondrially synthesized proteins that are probably not processed, it is not known whether the formyl group is removed (Horvath *et al.*, 2000a, b). Here we show that the *T. brucei* genome encodes two distinct putative PDF orthologues. One of the proteins was recombinantly expressed and exhibited deformylase activity. Furthermore, as predicted for deformylases, both *T. brucei* PDF orthologues are localized in mitochondria and required for efficient oxidative phosphorylation (OXPHOS), a process that in part depends on mitochondrially encoded proteins.

Results

Two PDF orthologues in T. brucei

The *T. brucei* genome encodes open reading frames (ORFs) for two putative PDF orthologues. The proteins have a predicted molecular weight of 30 kDa (TbPDF1: accession numbers Tb11.01.6120 and AF177768] and 42 kDa (TbPDF2: accession number Tb09.211.0190] respectively. They contain the three signature sequences that define the PDF protein family (Giglione and Meinnel, 2001b; Meinnel, 2000), even though motif 1 lacks the conserved glutamine residue that appears to be crucial for the recognition of the formyl group by TbPDF1 (Meinnel *et al.*, 1997) (Fig. 1). The two *T. brucei* PDFs share only a limited identity of 30% that is restricted to the C-terminal half of TbPDF1. However, both proteins have conserved orthologues in *Leishmania major* and *T. cruzi* with which they share an overall identity of 63% (for TbPDF1) and

75% (for TbPDF2). Interestingly, unlike the TbPDF2, the first PDF signature sequence of the *L. major* and the *T. cruzi* TbPDF2 orthologues includes the conserved glutamine residue of motif 1 (Fig. 1). The closest non-trypanosomatid orthologues that are identified by BLAST analysis are the PDF1B of *Arabidopsis thaliana* (for TbPDF1) and the PDF of the bacteria *Leptospira interrogans* (for TbPDF2). Finally, compared with bacterial PDFs, trypanosomatid PDF orthologues have both an N- and a C-terminal extension.

Biochemical analysis of recombinant TbPDF1

Based on changes in the conserved motifs, it has been suggested in a recent phylogenetic analysis that trypanosomal and archaeal PDFs form a separate group, termed type 3 PDFs (Giglione *et al.*, 2004). Thus, in order to test whether the trypanosomal PDF orthologues have PDF activity, we prepared recombinant TbPDF1, the more conserved of the two trypanosomal PDFs. To do so, full-length TbPDF1 and three variants thereof lacking either the first 30 amino acids (TbPDF1/31–260), the last 51 amino acids (TbPDF1/1–209) or both (TbPDF1/31–209) were expressed in *Escherichia coli* (Fig. 2A). All proteins carried a hexahistidine tag at their C-termini. Analysis of crude extracts with anti-hexahistidine tag antibodies showed that, whereas TbPDF1/31–260 was expressed at high levels, only low amounts of the other TbPDF1 variants accumulated (not shown). Thus, the presence of the N-terminal 30 amino acids and the absence of the C-terminal 51 amino acids had a deleterious effect on TbPDF1 expression.

Using nickel column affinity chromatography, the highly expressed TbPDF1/31–260 variant was purified to homogeneity as illustrated by the Coomassie-stained gel in Fig. 2B. By the same method, we obtained highly enriched fraction for full-length TbPDF1 and the TbPDF1/31–209 variant. Expression of the TbPDF1/1–209 variant was very weak. The corresponding nickel column eluate was poorly enriched in recombinant protein as a result, and contained a major contaminant that co-migrates with the recombinant protein as evidenced by immunoblot analysis using anti-Tag antibodies (Fig. 2B, lane TbPDF1/1–209).

Previous work had indicated that the catalytic core of bacterial PDFs is highly resistant to trypsin digestion (Meinnel *et al.*, 1996). Thus, we expect TbPDF1 to behave the same way. The predicted catalytic core of TbPDF1, encompassing approximately residues 36–198, is present in all four recombinant TbPDF1 variants. Consistent with this, low concentrations of trypsin were sufficient to convert full-length TbPDF1 into a trypsin-resistant fragment of approximately 28 kDa (Fig. 2C, top panel). The fragment still reacted with the anti-His-tag antibodies,

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TbPDF1 -----
LmPDF1 -----
TcPDF1 -----
TbPDF2 -----
LmPDF2 MSCITSPQPQSSPPRMDAHTCVICIGITMPPAARQRKGARRSLTLPPLLSLPWRKIRDQEGG
TcPDF2 -----

TbPDF1 -----
LmPDF1 -----
TcPDF1 -----MR
TbPDF2 -----
LmPDF2 AVCAGFPVQLPWKVRAEAALQSRFSPPAIRRRYLALSLPMRTSAAAAVAAAAHAGVRAIH
TcPDF2 -----MW

TbPDF1 ----MLRHLFRCTAAWAPKRSA-----
LmPDF1 RCVPWRRLLVCGGTSLLRGGVDAGG-----
TcPDF1 ----MLSRLSRTVPLLGPRRSA-----
TbPDF2 --MRIHHAFFRQSCRIMRGIDSAR-----STPTFVNGTDGGMGTI-----H
LmPDF2 TATGVAGNSCGASPRATVALPHSGLTLPSFTRCGASSAAFITRAHSVGGCCGCAAAVKQH
TcPDF2 TRSCIRCTFFCSSLYRKM-GLFPRR-----NDACRPALRAALCCS-----T

TbPDF1 -----TREQR--LKERVAARMELEAQVKS RVACYPHRSLSLTPALRLERHQVNTPLF
LmPDF1 -----APLSSSPS--FKETVEGKLEKEAEALRRVACYPHRSLSLTPALRLERHQVNTPLF
TcPDF1 -----AREKR--LKDRVAAQLELEAQVKS RVACYPHRSLSLTPALRLERHQVNTPLF
TbPDF2 MLFSKRTHTSNTTH--SNADSYMPGNSLHVEHPIVQLPARSLWCKQYPLDISLVRGEEF
LmPDF2 RLYSSHGCSNSSSGFSADSSRPYVPGQAVQETYPVQLPARSLWCKQYALDARRVAQGEY
TcPDF2 RLNSRRTAYDNNN--AAGEAAYMAGNTVHVEHPIVQLPARSLWCKQYPLDPEQVRSRGGF

TbPDF1 HSQLLNLNKMATDLCISFSAPKGHWDAAIVLIKG-----HPNEANFEV
LmPDF1 MSSLMDLNQLATGLHCLFSAPKRAHWDAAVILIKS-----NPDETEYEV
TcPDF1 QSQLLSLKMASDLRCLFSAPKGHWDATVVLIKG-----HPDEEVFEV
TbPDF2 NDMIGRVRARHYYQYPSLCAPIGNNVQMPFLFDGTVFINPINLDALEVEEIAKQKSI
LmPDF2 AGLISQVREARHYYQYPSMSAPQIGNNVQMPFLFDNSVFINPNLDEVEVRAEAARQGM
TcPDF2 AEMIERVRAARQYQYPSLCAPIGNNVQMPFLFDGTVFINPNLLELEAAGSRSGMP

TbPDF1 WVNPTVPGYDDRHSAIPMYGMWENCISCGACTAWVIRPQSITCSGLDEYENKTEVLDGM
LmPDF1 WVNPSVPGYDDRNAVAPMYGMWENCISCGTATAWVIRPQRITCSGYDEHGNHKVQLDGM
TcPDF1 WVSPSVPYDARTSIAIPMYGMWENCISCGATAAWVIRPQCVCSCGWDEYENKTELLDGM
TbPDF2 FVEAEALWMEKKRRREGKTCFAWEPSCASCCFLHFIERPSTVRVRAIAEDGNPFVTLDKM
LmPDF2 WVAFEEEMRELAEGLTGFWEPSCASSGFLHYIERPLTVMRRALEHCKPFTVTLDKM
TcPDF2 IAEAEAQWVASCRRREGKTCFAWEPSCASCCFLMHYIERPATVRIRAIAGADHPFVTLKEM

TbPDF1 RARCLMHELDHLSGRTILDQAQGFPEFVSGIANGQRDLWPPNPFSAEAYMTSPHQFFD--
LmPDF1 RARCLMHELDHLMGKTIIPHQAVGPEFVSSVAMAQRYLWPNANPFSAEAYVTPGQFFD--
TcPDF1 RARCLMHELDHLGKTIILHQALGPEFVSGIANGQRDLWPPNPFSAEAYVTPHQFFD--
TbPDF2 RARMALHELDHLNGILFTRRIPTDTHVVPLEGFSTMSWDDYPSLEARSTFLYTIPTFP
LmPDF2 RARMALHELDHLQGVLFTRRVVDTHVVPMEGFVTMSDWSDDYPSLEARSTFLYTIPTFP
TcPDF2 RARMALHELDHLQGVLFTRRIPTDTHVVPLEGFSTMSWDDYPSLEARSTFLYTIPTFP

TbPDF1 --YVKNGPIIPPGEWYFAQSMNQFEDARLSH-----
LmPDF1 --YVQNETVIPPGEWYFAQNVREFFSNEQIQ-----
TcPDF1 --YVKNIPIVPPGEWYFAQNMQQHFQDARLN-----
TbPDF2 YTEITDSVEDAQLLDRKFEDGIYPGCEHRRMRIESAAFEELQRAQWRMEKTRREENSEY
LmPDF2 YHFVSDAVPDGNLLDRKFEDGIYPGCEQDRQRIELTAMEIQRNVWRMEKAKRKEG-GQ
TcPDF2 YTEITDNVEDAQLLDRKFEDGIYPGCEHRRMRIESAALEELQRAQWRMEKACSEEGDST

TbPDF1 -----
LmPDF1 -----
TcPDF1 -----
TbPDF2 DGVEGEVDEEDQEHCETAAGGVAVKDNAPQ
LmPDF2 QCGRGDVTAVE---DDDGSGAAGAR---
TcPDF2 QCMR-DTDEEQHEDEETEEDVTAQKGTL--

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Fig. 1. *Trypanosoma brucei* contains two PDF orthologues. Multiple sequence alignment of the TbPDF1 and TbPDF2 and their corresponding orthologues in *Leishmania major* (LmPDF1, LmPDF2) and in *Trypanosoma cruzi* (TcPDF1, TcPDF2). The sequences were aligned using the CLUSTAL W program with default parameters. Strictly conserved residues and conservative replacements are shown in black and grey boxes respectively. The three PDF signature sequences (Giugliano and Meinel, 2001b) are indicated in the three large boxes.

indicating that its C-terminus was intact. TbPDF1/31–260 co-migrated with the proteolytic fragment of the full-length enzyme and resisted proteolytic attack within the same range of concentration (Fig. 2C, middle panel). In contrast, TbPDF1/31–209 (Fig. 2C, bottom panel) and TbPDF1/1–209 (not shown) were completely hydrolysed at very low concentrations of trypsin. These experiments show that TbPDF1 has a protease-resistant core similar in size to the one found in bacterial PDFs. Furthermore, the removal of the N-terminus at very low trypsin concentrations suggests that, in agreement with previous analyses of PDFs from other organisms, this extension has no defined secondary structure.

Finally, full-length recombinant TbPDF1 and the most stable truncated variant TbPDF1/31–260 were assayed for deformylation activity. Both proteins were able to remove the formyl group from formylated peptides consisting of two or more amino acids in length (Table 1).

Highest activities were observed for dipeptides containing either formylated methionine or phenylalanine. Interestingly, however, a dipeptide containing isoleucine, even though it is also hydrophobic in nature, was a much worse substrate (Table 1). Thus, the substrate specificity of TbPDF1 is identical to that of bacterial PDFs, which also discriminate between phenylalanine and isoleucine (Ragusa *et al.*, 1999).

The observed specific activity of TbPDF1 was very low ($k_{cat}/K_M = 8 \text{ M}^{-1} \text{ s}^{-1}$) but similar to the one observed for human (Serero *et al.*, 2001; Serero *et al.*, 2003; Lee *et al.*, 2004) and *Plasmodium falciparum* PDFs (Bracchi-Ricard *et al.*, 2001) when tested with comparable substrates. Specific activities of the same order of magnitude have also been described for some bacterial PDFs. In these cases, the low activities could be explained by an exchange of the metal cation found in the active site *in vivo* for another less efficient one that may render the

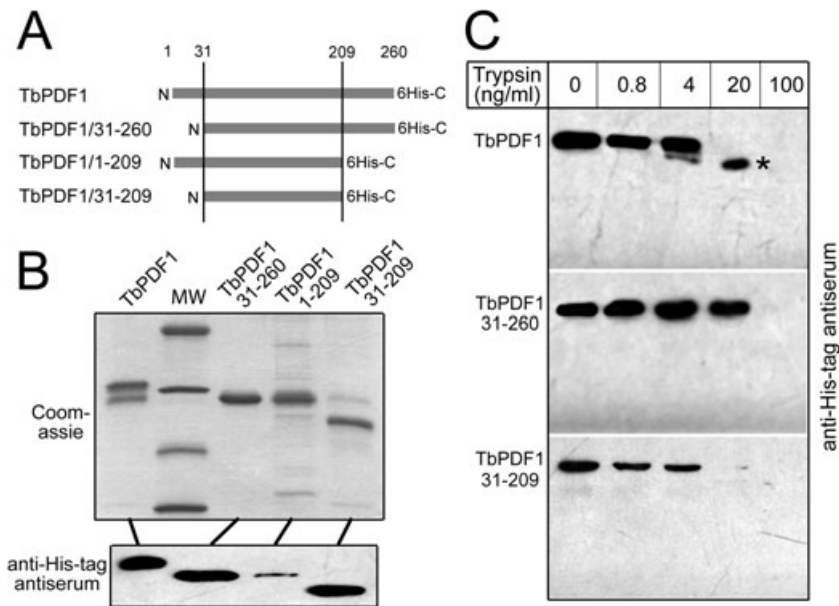


Fig. 2. Biochemical analysis of recombinant TbPDF1.

A. Schematic representation of the 6xHis-tagged TbPDF1 variants that were recombinantly expressed in *E. coli*.

B. Upper panel, Coomassie-stained 12% SDS polyacrylamide gel containing approximately 2 µg each of Ni-NTA agarose-purified pool containing the recombinant proteins. In the case of TbPDF1/1–209, purity was assessed to be less than 10% because of poor overexpression and co-elution with many contaminating proteins, including a major one co-eluting with most variants. The molecular weight markers in the second lane correspond to 43, 30, 20.1 and 14.4 kDa. Lower panel, immunoblot analysis using anti-His-tag antibodies of a duplicate gel containing the same samples as in the upper panel.

C. Proteolytic digestion of the recombinant TbPDF1 variants with the indicated concentrations of trypsin (ng ml⁻¹). Proteins were detected by immunoblot analysis using anti-His-tag antibodies. The asterisk shows the trypsin-resistant core of full-length TbPDF1 that in size corresponds to trypsin-resistant TbPDF1/31–260 seen in the middle panel.

enzyme unstable (Ragusa *et al.*, 1998). Thus, this may also apply to TbPDF1, as measurements of the metal content show that zinc is present (0.6 ± 0.2 mole mole⁻¹ PDF) in all four variants of the protein. In analogy with results in *E. coli* (Meinzel and Blanquet, 1995), this indicates that zinc may have replaced another more efficient cation that is used *in vivo* (Ragusa *et al.*, 1998). Unfortunately, as previously reported for human PDF (Lee *et al.*, 2004), all attempts to optimize the activity by using oxidant scavengers, or by replacing zinc by other cations, were unsuccessful. Furthermore, we cannot exclude that imidazole elution from the nickel column could participate in enzyme inactivation via metal cation removal.

Finally, as the normally conserved glutamine in motif 1 of TbPDF1 is substituted by a lysine (Fig. 1), we have also expressed and purified a TbPDF1 variant where the conserved glutamine was reintroduced. However, the resulting TbPDF1 variant was not more active in deformylation than the wild-type one.

In summary, these results show that TbPDF1, similar to the PDF of humans and *P. falciparum*, has deformylase activity. Furthermore, as for other eukaryotic PDFs, the activity is not dependent on the presence or absence of the first 30 N-terminal residues.

Intracellular localization of TbPDF1 and TbPDF2

Both trypanosomal PDF orthologues are predicted to have mitochondrial targeting signals. However, in *T. brucei* such predictions are difficult, as mitochondrial presequences can be very short (Häusler *et al.*, 1997). Thus, in order to determine the localization of the two proteins, we prepared transgenic procyclic *T. brucei* cell lines, allowing inducible expression of TbPDF1 and TbPDF2 versions that carry the Ty1-peptide as a C-terminal epitope tag (Bastin *et al.*, 1996). Immunoblot

Table 1. Substrate specificity of recombinant TbPDF1.

Peptide ^a	Relative deformylation rate ^b
Fo-Met-X	100
Fo-Phe-X	40
Fo-Ala-X	2
Fo-Ile-X	3
Fo-Met	<< 1

a. X indicates that the peptide is composed of an equimolar mixture of 19 usual amino acids.

b. Deformylation rates were measured using the TbPDF1/31–260. A value of 100 was given to the highest value. It corresponds to a deformylation rate of 13.5 pmole of hydrolysed peptide per minute and per mg protein added (0.008 s⁻¹). Standard deviation was < 20%.

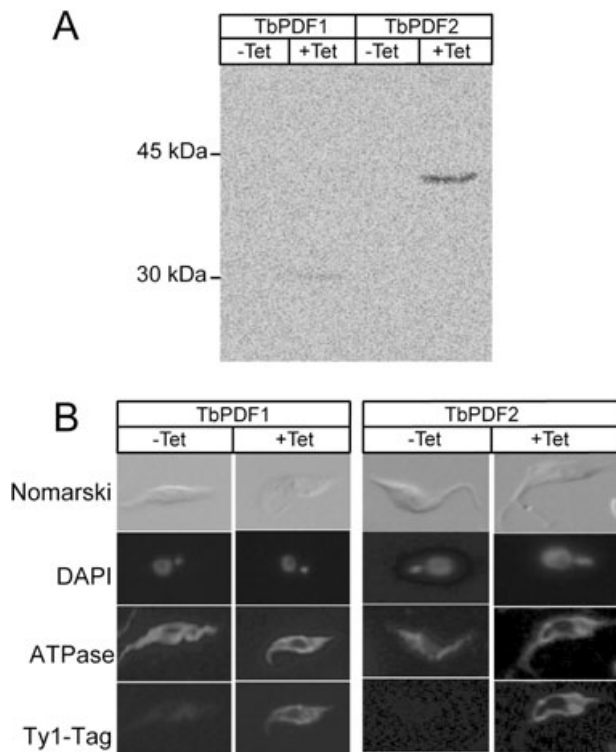


Fig. 3. Intracellular localization of TbPDF1 and TbPDF2. Localization of trypanosomal PDF orthologues. A. Immunoblot analysis of total cellular extracts of two transgenic *T. brucei* cell lines expressing TbPDF1 and TbPDF2 carrying Ty1-tags at their C-termini. Expression is under the control of the tetracycline inducible (–, +Tet) procyclin promoter. The Ty1-tag was detected using the monoclonal BB2 antibody. B. Immunofluorescence analysis of the same cell lines shown in A. The top two lines of each panels show the Nomarski picture and the DNA-staining using DAPI. The lower two lines of the panels depict the staining seen by using a polyclonal antibody against a subunit of the ATPase, which serves as a mitochondrial marker, and the staining obtained by using the Ty1-tag-specific monoclonal BB2 antibody.

analysis using anti-Ty1 antibody showed that, in both induced cell lines, a single band was detected that corresponded to the expected molecular weights of TbPDF1 and TbPDF2 respectively (Fig. 3A). Immunofluorescence of either of the two cell lines revealed a reticulate staining pattern, specific for induced cells, that is identical to the one seen for the mitochondrial marker (Fig. 3B). Thus, these results show that both TbPDF1 and TbPDF2 are localized in mitochondria.

Effect of RNAi-mediated ablation of TbPDF1 and TbPDF2 on growth

In order to study the importance of TbPDF1 and TbPDF2 for growth and survival of procyclic *T. brucei*, we established transgenic cell lines that allow individual or combined RNAi-mediated ablation of the two enzymes. Growth of the RNAi cell lines was tested (i) in the standard

medium SDM-79 (Brun and Schönberger, 1979), which contains proline and glucose as the major energy sources (Fig. 4A and B), as well as (ii) in SDM-80 (Lamour *et al.*, 2005), a modified version of SDM-79 that lacks glucose (Fig. 4C).

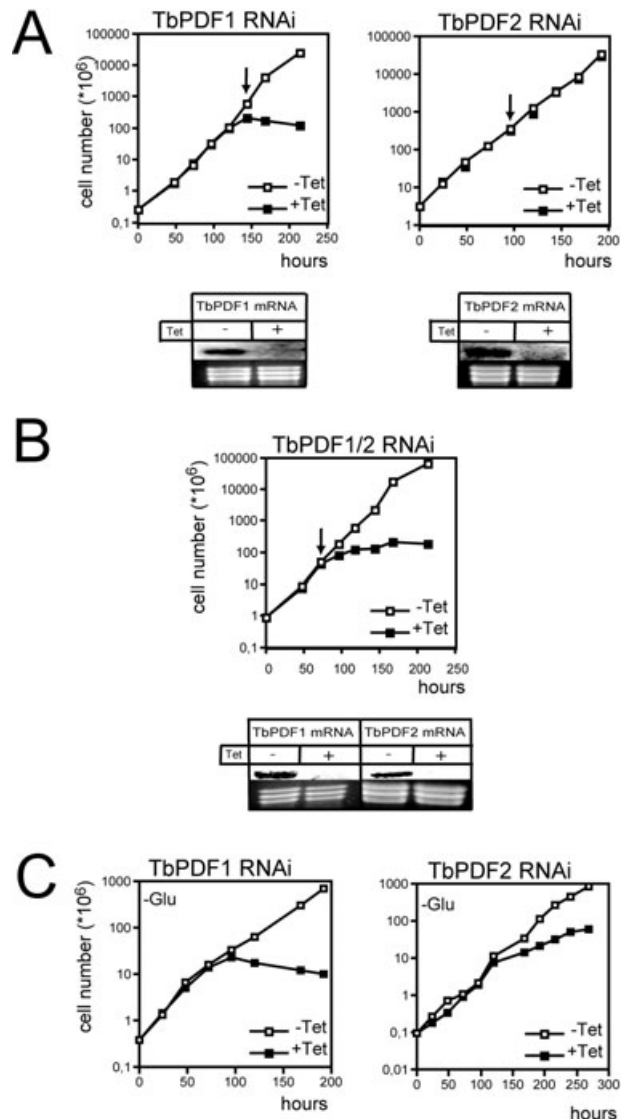


Fig. 4. Effect of RNAi-mediated ablation of TbPDF1 and TbPDF2 on growth.

A. Growth curves in standard culture medium SDM-79 of uninduced and induced (–, +Tet) clonal TbPDF1 (left panel) and TbPDF2 (right panel) RNAi cell lines. The panels below the graphs show Northern blots for the corresponding TbPDF mRNAs. The rRNAs in the lower panel serve as loading controls. Time of sampling is indicated by the arrows.

B. Growth curve in standard culture medium SDM-79 of an uninduced and induced (–, +Tet) culture of a representative clonal TbPDF1/TbPDF2 double-RNAi cell line. The panel below the graph shows a Northern blot for the two TbPDF mRNAs.

C. Growth curves of uninduced and induced TbPDF1 and TbPDF2 RNAi cell lines in culture medium SDM-80 that lacks glucose (– Glu). All growth curves have been determined at least three times in independent experiments and were shown to be highly reproducible.

Ablation of TbPDF1 caused a growth arrest in SDM-79 after approximately 150 h of induction (Fig. 4A, left graph). However, when cells ablated for TbPDF2 were tested in the same medium, growth was not affected. Thus, TbPDF2, in contrast to TbPDF1, appears to be dispensable for growth on SDM-79. However, a double-RNAi cell line allowing simultaneous ablation of TbPDF1 and TbPDF2 (Fig. 4B) shows a growth arrest after approximately 70 h of induction, much earlier than the 150 h that is needed if only TbPDF1 is ablated. These results indicate that, whereas a reduced level of TbPDF2 alone does not affect growth in SDM-79, it causes the growth arrest induced by ablation of TbPDF1 to occur much earlier.

When tested in glucose-free SDM-80 medium, the growth arrest caused by ablation of TbPDF1 occurs already approximately 90 h after induction (Fig. 4C, left panel). Furthermore, in SDM-80, unlike in SDM-79, a slow growth phenotype is also detected for the induced TbPDF2 RNAi cell line (Fig. 4C, right panel).

In procyclic *T. brucei*, glucose, after conversion into pyruvate, is mainly used for mitochondrial substrate-level phosphorylation (SUBPHOS) in the trypanosome-specific acetate:succinate CoA transferase/succinyl-CoA synthetase (ASCT) cycle (Van Hellemond *et al.*, 1998) and, not as might be expected, fed into the citric acid cycle (Bochud-Allemann and Schneider, 2002; van Weelden *et al.*, 2003). When grown in SDM-79, where glucose is available, the energy needs of *T. brucei* can be fulfilled mainly by SUBPHOS. In the glucose-free SDM-80 medium, however, the sole energy source is proline that can only be utilized by OXPHOS. Growth in SDM-80 therefore selects for cells capable of performing efficient OXPHOS (Lamour *et al.*, 2005). Thus, the fact that ablation of either of the two proteins causes a stronger growth phenotype in glucose-free than in the standard medium suggests that TbPDF1 and to a more limited extent also TbPDF2 are, at least indirectly, required for efficient OXPHOS.

Effect of RNAi-mediated ablation of TbPDF1 and TbPDF2 on mitochondrial ATP production

The proteins encoded in the mitochondrion of *T. brucei* – the substrates of TbPDF1 and possibly TbPDF2 – either function for the most part directly in OXPHOS, or are components of the mitochondrial translation machinery that produces them (Feagin, 2000). Thus, should removal of the formyl group be essential for the function of any of these mitochondrially encoded proteins, the lack of PDF activity should ultimately affect OXPHOS. The mitochondrion of procyclic *T. brucei* produces ATP, by OXPHOS as well as by SUBPHOS linked to the citric acid cycle or the ASCT cycle (Besteiro *et al.*, 2005). Using isolated mito-

chondria, we have recently established an assay that allows quantification of both modes of ATP production (Bochud-Allemann and Schneider, 2002; Schneider *et al.*, 2007). In these assays, OXPHOS is induced by the addition of either succinate or glycerol-3 phosphate. As expected, this type of ATP production is antimycin sensitive. Mitochondrial SUBPHOS, which is resistant to antimycin, is induced by the addition of α -ketoglutarate (for the SUBPHOS in the citric acid cycle) or pyruvate (for the SUBPHOS in the ASCT cycle). In the latter case, succinate has to be added as a co-substrate. Thus, 25–50% of the ATP produced by the combination of pyruvate and succinate is due to OXPHOS and therefore antimycin sensitive (Bochud-Allemann and Schneider, 2002). Isolated mitochondria of *T. brucei* are depleted for nucleotides; therefore, ADP has to be added in all assays. Atractyloside treatment prevents mitochondrial import of the added ADP and serves as a control for all modes of mitochondrial ATP production. Figure 5A shows that, in line with the deformylase activity of the recombinant protein, ablation of TbPDF1 results in a loss of OXPHOS induced either by glycerol-3 phosphate or by succinate, but does not affect mitochondrial SUBPHOS assayed by pyruvate or α -ketoglutarate respectively. Ablation of TbPDF2, on the other hand, does not influence either mode of ATP production (Fig. 5B). Thus, TbPDF1, but not TbPDF2, is required for OXPHOS in isolated organelles. These results are in agreement with the observation that TbPDF1 is required for growth in any medium, and explain why the growth arrest during TbPDF1 RNAi appears earlier in glucose-free than in the standard medium (Fig. 4). Isolated mitochondria ablated for TbPDF2 are not impaired in OXPHOS, which is consistent with the normal growth of the induced RNAi cell line that is observed in standard medium. However, ablation of TbPDF2 reinforces the growth arrest of cells that are already ablated for TbPDF1 and leads to a slow growth phenotype in glucose-free medium (Fig. 4). This suggests that while TbPDF2 is not required for the level of OXPHOS that is needed under normal growth conditions, it is nevertheless required for the highly efficient OXPHOS that is demanded in glucose-free medium.

Discussion

A phylogenetic analysis based on alignments of the three conserved motifs suggests that the trypanosomal PDFs are most closely related to the PDFs of Archaea (Giglione and Meinnel, 2001b). In fact, archaeal and trypanosomatal PDFs are sufficiently different from all other PDFs to classify them in an own group, termed type 3 PDFs (Giglione *et al.*, 2004). Archaea do not use formyl-methionine to initiate protein synthesis; thus, it is not known which reaction archaeal PDF orthologues catalyse. Interestingly,

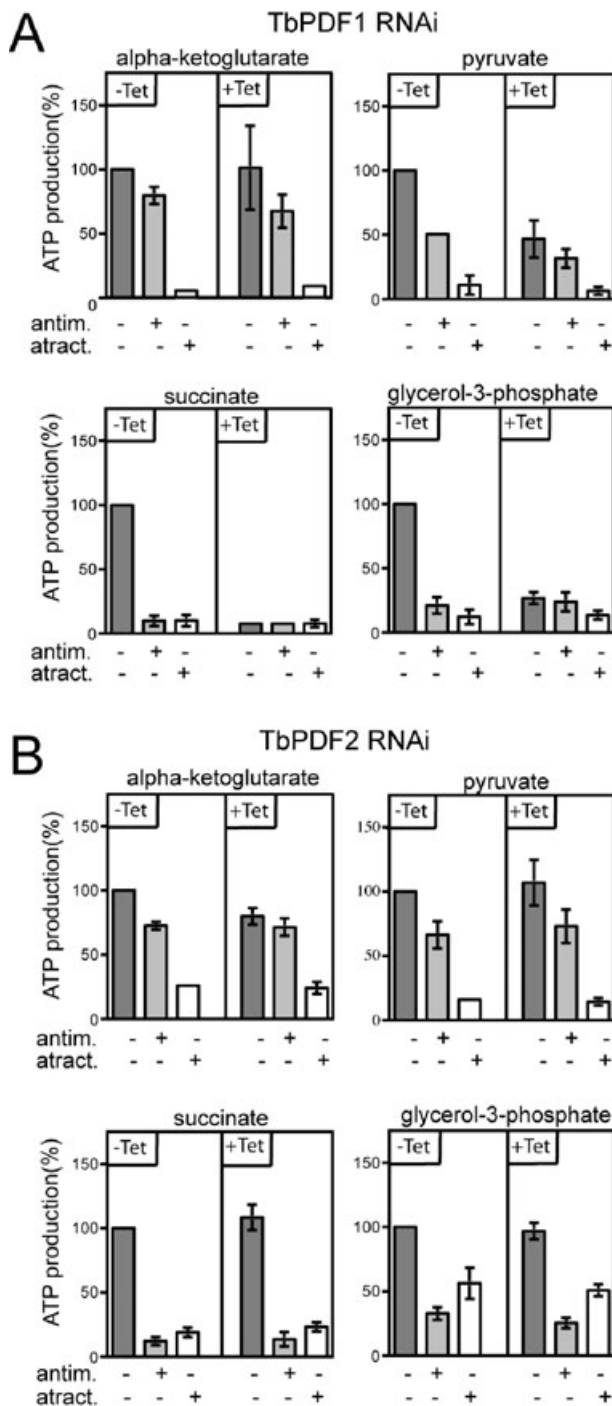


Fig. 5. Effect of RNAi-mediated ablation of TbPDF1 and TbPDF2 on mitochondrial ATP production. OXPHOS, induced by succinate or glycerol-3 phosphate, and SUBPHOS, induced by α -ketoglutarate or pyruvate, were assayed in crude mitochondrial fractions of TbPDF1 (A) and TbPDF2 (B) RNAi cell lines respectively. Uninduced cells (- Tet) are shown on the left, induced cells (+ Tet) on the right of each panel. The substrate tested is indicated at the top and additions of antimycin (antim.) and atractyloside (atract.) at the bottom of each panel. ATP productions in mitochondria isolated from uninduced cells tested without antimycin or atractyloside are set to 100%. The bars represent means expressed as percentages from three or more independent inductions. Standard errors are indicated.

however, recombinant PDF1 of *T. brucei*, a type 3 enzyme, exhibits deformylase activity whose specificity mirrors that of bacterial enzymes. The specific activity of the recombinant protein is low but comparable to that of human and *P. falciparum* PDFs that belong to class 1 A and 1B respectively (Giglione *et al.*, 2004). Other features that are shared between TbPDF1 and the well-characterized bacterial PDFs are a trypsin-resistant active core of similar size and the capability to bind metals. In fact, it is likely that an exchange of a metal ion, of yet unknown nature, for zinc in the recombinant enzyme is responsible for the low specific activity that is observed.

We have previously established that active MTF is present in the mitochondrion of *T. brucei* (Tan *et al.*, 2002). Unexpectedly, due to the absence of mitochondrial tRNA genes in *T. brucei*, the tRNA that becomes formylated is the elongator tRNA^{Met} imported from the cytosol. The proteins encoded on the *T. brucei* mitochondrial DNA are subunits I–III of cytochrome oxidase, cytochrome b, subunit 6 of the ATPase, the ribosomal protein Rps12, and at least seven subunits of the NADH dehydrogenase (Feagin, 2000). When translated, the corresponding nascent proteins will necessarily carry a formyl-methionine at their N-termini, because of mitochondrial MTF activity. Thus, the only compartment in *T. brucei* where substrates for PDFs are present is the mitochondrion. In agreement with this, TbPDF1 is localized to mitochondria. However, up to now the N-termini of only two mitochondrially synthesized proteins, cytochrome b and cytochrome oxidase subunit I (Horvath *et al.*, 2000a, b), have directly been sequenced. Both of these proteins had to be treated with HCl prior to sequencing because their N-termini were blocked, suggesting that they consisted of an unprocessed formyl-methionine. Thus, *in vivo* evidence for deformylation of mitochondrially encoded proteins is still lacking, as is also the case for human mitochondrial proteins (Serero *et al.*, 2003).

Isolated mitochondria from TbPDF1-ablated cells show a specific loss of OXPHOS, but not of SUBPHOS. In agreement with this, the growth arrest induced by ablation of TbPDF1 occurs much earlier in glucose-free medium, that selects for efficient OXPHOS, than in the standard medium that provides substrates for OXPHOS and mitochondrial SUBPHOS. The substrates of PDFs are mitochondrially encoded proteins, many of which are required for OXPHOS but not for mitochondrial SUBPHOS. These results therefore support the idea that TbPDF1 is a deformylase.

Unexpectedly, unlike humans (Nguyen *et al.*, 2003; Serero *et al.*, 2003; Lee *et al.*, 2004) but similar to plants (Giglione *et al.*, 2000), *T. brucei* has two PDF homologues, both of which are localized in mitochondria. In contrast to TbPDF1, TbPDF2 is dispensable for growth on standard medium. Interestingly, however, TbPDF2-

ablated cells show a slow growth phenotype on glucose-free medium, suggesting that the function of TbPDF2 is also, at least indirectly, linked to OXPHOS. Furthermore, the synergistic growth phenotype observed in the cell line ablated for both trypanosomal PDFs suggests that the two proteins are functionally connected.

Given the strong homology of the catalytic domain of TbPDF2 with other well-characterized PDFs (Giglione and Meinel, 2001b), it appears likely that it also has deformylase activity. Thus, TbPDF1 and TbPDF2 may in part have overlapping functions: TbPDF1 may deformylate all mitochondrially synthesized proteins, although some only inefficiently, whereas TbPDF2 might be more selective and only deformylate proteins that are poorly recognized by TbPDF1. However, attempts to purify recombinant TbPDF2 have so far been unsuccessful. Furthermore, overexpression of TbPDF2 did not rescue the growth arrest that is observed in the induced TbPDF1 RNAi cell line. The described scenario is therefore mainly based on speculation, and the function of TbPDF2 remains unknown at present.

Why are there two PDF orthologues in *T. brucei*? Interestingly, plants also have two of these enzymes (Giglione *et al.*, 2000). One is essentially localized in mitochondria (PDF1A), whereas the other (PDF1B) is found in both plastids and mitochondria. Unlike PDF1A, PDF1B is essential for chloroplast function (Giglione and Meinel, 2001a). Nevertheless, the two proteins are in part functionally redundant. Hence, overexpression of PDF1A partially compensates for the absence of PDF1B in chloroplasts, and overexpression of PDF1A can fully complement the lack of PDF1B (Giglione *et al.*, 2003). Plant PDFs are believed to mainly differ by their capacity to bind distinct metal cation, Zn²⁺ for PDF1A and Fe²⁺ for PDF1B (Serero *et al.*, 2001). Consequently, a unique feature of PDF1B is its strong sensitivity to oxidation. This led to the proposal that PDF1A was suited for environments prone to oxidative stress such as mitochondria (Serero *et al.*, 2001). Trypanosomatids are closely related to the euglenids, most of which have a plastid. Furthermore, a number of plant-like genes encoding homologues of proteins found in chloroplasts have been found in the genome of *T. brucei* (Hannaert *et al.* 2003). Thus, it has been suggested that *T. brucei* may once have had a plastid which it has lost secondarily (Martin and Borst, 2003). It is therefore likely that one of the two trypanosomal PDFs represents an evolutionary remnant of a PDF that originally was also targeted to the plastid. However, even if the evolutionary history of one of the trypanosomal PDFs is linked to plastids, it is clear that today both proteins have a mitochondrial function. TbPDF1 is strictly required for OXPHOS under all conditions. The requirement of TbPDF2 is less stringent but is seen when growth is tested in glucose-free medium or at limiting concentra-

tions of TbPDF1. Finally, like in plants, it is not unlikely that the nature of the metal cation bound *in vivo* could be different between the two trypanosomal PDFs, each being more adapted to unique environmental stress conditions.

Recently, a PDF orthologue has been identified and characterized in *P. falciparum* (Meinel, 2000; Bracchi-Ricard *et al.*, 2001). PDF inhibitors were shown to interfere with growth of the intraerythrocytic stage of the parasite (Bracchi-Ricard *et al.*, 2001; Wiesner *et al.*, 2001). Based on these observations, it has been proposed that the plasmodial PDF could be exploited as a novel target for antimalarial drugs (Meinel, 2000; Giglione and Meinel, 2001b). The PDF orthologues of *T. brucei* are essential for mitochondrial function, and their sequence is quite divergent from other eukaryotic PDFs. This suggests that also the trypanosomal PDFs may be suitable drug targets. Thus, it is possible that the trypanosomal PDFs are required for processing of subunit 6 of the ATPase, a mitochondrially encoded protein that was demonstrated to be essential for both life-cycle stages (Schnauffer *et al.*, 2005). Unlike *T. brucei*, for which OXPHOS is specific for the insect stage and does not occur in the bloodstream form of the parasite (Durieux *et al.*, 1991; Schneider, 2001), *Leishmania* and *T. cruzi* need a functional respiratory chain for survival in their respective vertebrate hosts (Van Hellemond and Tielens, 1997). Thus, in *T. cruzi* and *Leishmania*, PDF orthologues appear to be excellent drug targets to fight the associated diseases.

Experimental procedures

Purification of recombinant TbPDF1 from *E. coli*

The ORFs corresponding to full-length TbPDF1 (Fig. 1) and the described variants thereof (Fig. 2) were cloned into pQE60 (Qiagen), a vector specialized for in-frame fusions with a C-terminal 6xHis tag. Transfected *E. coli* M15 was grown at 30°C in 2xTY containing 50 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ kanamycin. Expression of the recombinant proteins was induced with 0.1 mM IPTG at an OD₆₅₀ of 0.9 for 4 h. Cells were harvested and resuspended in 20 mM Tris-HCl pH 8.0, 20 mM 2-mercaptoethanol, 0.3 M NaCl (buffer A) containing 10 mM imidazole. The sample was sonicated, and cell debris removed by centrifugation. TbPDF1 recombinant proteins were purified by affinity chromatography on Ni-NTA Agarose columns (Qiagen). The columns were washed with an excess of buffer A containing 20 mM imidazole, and the His-tagged proteins were eluted with a linear 20–110 mM imidazole gradient in buffer A. The eluted proteins were dialysed against 20 mM Tris (pH 8.0), 1 mM 2-mercaptoethanol containing 55% glycerol and stored in aliquots at -30°C. Purity was assessed to be 70% (TbPDF1), 95% (TbPDF1/31–260), 10% (TbPDF1/1–209) and 60% (TbPDF1/31–209).

Biochemistry

Peptide deformylase (PDF) activity assays were carried out in 50 mM HEPES (pH 7.0) at 37°C for 90 min as described

(Meinzel and Blanquet, 1995). Proteolytic digestion with different concentrations of trypsin (Sigma) of the indicated PDF variants (Fig. 2) was performed in 0.1 M Tris-HCl (pH 7.7) containing 1 mM 2-mercaptoethanol. Samples were incubated at 37°C for 15 min, and the reaction was stopped by the addition of chicken egg-white ovomucoid 2/1 (w/w) with respect to trypsin. Finally, the reaction products were analysed by 12% SDS-PAGE. To measure zinc content by atomic absorption spectroscopy, protein samples were dialysed overnight against a buffer containing 20 mM Tris-HCl (pH 7.5) containing 0.1 M KCl. Atomic absorbencies were measured at 213.9 nm in the peak height mode during 5 s after 0.1 ml injections using a Varian AA220 spectrophotometer equipped with an air-acetylene burner (Serero *et al.*, 2003). Zinc concentrations in serial dilutions of the enzyme samples were calculated by comparison with standard solutions of known concentrations.

Production of transgenic *T. brucei* cell lines

As a tag to localize TbPDF1 and TbPDF2, a 10-amino-acid epitope of the major structural protein of the yeast Ty1 virus-like particle was used which is recognized by the monoclonal antibody BB2 (Bastin *et al.*, 1996). The sequences corresponding to the C-terminal Ty1-tagged proteins were cloned into a derivative of the plasmid pLew-100, which allows tetracycline inducible expression of the tagged proteins (Wirtz *et al.*, 1999). RNAi of TbPDF1 and TbPDF2 was performed using stem-loop constructs containing the phleomycin-resistance gene as described (Bochud-Allemann and Schneider, 2002). As inserts we used a 715 bp fragment (nucleotides 65–779) of the TbPDF1 gene and a 644 bp fragment of the TbPDF2 gene (nucleotides 2–645). To obtain the double-RNAi cell lines analysed in Fig. 4B, we replaced the phleomycin-resistance gene of the construct used to ablate TbPDF2 with the puromycin-resistance gene, and used the resulting plasmid to transfect the previously characterized single knock-down for TbPDF1. Transfection of *T. brucei*, selection with antibiotics, cloning and induction with tetracycline were conducted as described (McCulloch *et al.*, 2004). All transgenic cell lines used in this study are based on *T. brucei* strain 29–13, which was grown in SDM-79 (Brun and Schönenberger, 1979) supplemented with 15% FCS, 50 µg ml⁻¹ hygromycin and 15 µg ml⁻¹ G-418. Growth of RNAi cells was also analysed in SDM-80, a modified SDM-79 that lacks glucose and which is supplemented with 10% of dialysed FCS as described (Lamour *et al.*, 2005).

In organello ATP production assays

ATP production assays were performed as described (Schneider *et al.*, 2007). In total, 10⁷ cell equivalents of the digitonin-extracted cells from the TbPDF1 RNAi cell line and the TbPDF2 RNAi cell line were used for each assay. Induction times for the TbPDF1 and TbPDF2 RNAi cell lines used for the experiments shown in Fig. 5 were 160 and 100 h respectively. However, for the TbPDF2 RNAi cell lines, the same results were obtained for longer induction times. To induce ATP production, 5 mM of the indicated substrates (succinate, glycerol-3 phosphate, α -ketoglutarate or

pyruvate/succinate) and 67 µM of ADP was added. After incubation at 27°C for 30 min, the reaction was processed and the ATP concentration determined as described. Inhibitors were preincubated with mitochondria for 10 min on ice and used at the following final concentrations: atractyloside (33.3 µg ml⁻¹) and antimycin (2.7 µM). ATP production was then induced by the addition of substrate and ADP.

Miscellaneous

RNA for Northern analysis was isolated by the acid guanidinium method (Chomczynski and Sacchi, 1987). The probes used were random hexamer α -³²P dCTP-labelled double-stranded DNA fragments corresponding to nucleotides 65–779 of the TbPDF1 gene and to nucleotides 2–645 of the TbPDF2 gene respectively. Immunofluorescence was performed as described (Schneider *et al.*, 1987).

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