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Characterization of Two Protein Disulfide Isomerases from the Endocytic Pathway of Bloodstream Forms of *Trypanosoma brucei**

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Proteins from the endocytic pathway in bloodstream forms of *Trypanosome brucei* are modified by the addition of linear poly-*N*-acetyllactosamine side chains, which permits their isolation by tomato lectin affinity chromatography. Antibodies against this tomato lectin binding fraction were employed to screen a cDNA expression library from bloodstream forms of *T. brucei*. Two cDNAs were prominent among those selected. These cDNAs coded for two putative protein disulfide isomerases (PDIs) that respectively contained one and two double-cysteine redox-active sites and corresponded to a single domain PDI and a class 1 PDI. Assays of the purified recombinant proteins demonstrated that both proteins possess isomerase activity, but only the single domain PDI had a reducing activity. These PDIs possess a number of unusual features that distinguish them from previously characterized PDIs. The expression of both is developmentally regulated, they both co-localize with markers of the endocytic pathway, and both are modified by *N*-glycosylation. The larger PDI possesses *N*-glycans containing poly-*N*-acetyllactosamine, a modification that is indicative of processing in the Golgi and suggests the presence of a novel trafficking pathway for PDIs in trypanosomes. Although generally PDIs are considered essential, neither activity appeared to be essential for the growth of trypanosomes, at least *in vitro*.

African trypanosomes, *e.g. Trypanosoma brucei*, are extracellular protozoan parasites, transmitted by tsetse flies, that cause sleeping sickness in humans and Nagana in cattle. The life cycle of trypanosomes involves stage-specific forms adapted for life in the mammalian host and the tsetse fly vector (1). Bloodstream forms of *T. brucei* evade the mammalian immune response primarily by antigenic variation of their surface coat, which consists of a single, tightly packed and highly immunogenic protein species (the variable surface glycoprotein) that covers their entire cellular surface. All variable surface glycoproteins exhibit a conserved pattern of cysteine residues that participate in disulfide bonds essential for the common rod-like structure of the protein (2). However, these unicellular eukaryotes depend on receptor-mediated uptake of host-derived factors, *e.g.* transferrin and lipoproteins, for growth. Bloodstream forms have high rates of endocytosis, whereas this activity in procyclic forms appears to be absent or significantly reduced (3–5). Endocytosis and exocytosis are restricted entirely to a small invagination of the plasma membrane at the base of the flagellum called the flagellar pocket that represents ~0.5% of the cellular surface (6, 7). Significantly, *N*-glycans containing linear poly-*N*-acetyllactosamine are associated only with proteins, including receptors for host ligands and invariant proteins, from the flagellar pocket/endocytic pathway (8). These glycoproteins bind specifically to tomato lectin, a property that has allowed isolation of at least 20 different proteins from bloodstream forms of *T. brucei.* Antibodies raised against the total tomato lectin binding fraction isolated from bloodstream forms were used to screen a bloodstream form cDNA expression library to select for genes that potentially code for proteins from the flagellar pocket/endocytic pathway. Interestingly, two cDNAs were abundant among the clones selected and appeared to code for two distinct protein disulfide isomerases (PDIs).[1] One of these cDNAs was originally identified 15 years ago by Hsu *et al.* (9) as part of their studies on gene expression in trypanosomes, but the putative PDI was not characterized. The PDIs are thiol-disulfide oxidoreductases that catalyze the formation, reduction, and isomerization of disulfide bonds, depending on the redox environment, and can also function as molecular chaperones, in which they assist in the folding of proteins (10–12). Here we describe the first detailed characterization of two PDIs present in the tomato lectin binding fraction from *T. brucei* bloodstream forms. Both these PDIs possess isomerase activity, are bloodstream stage-specific, and co-localize with the endocytic pathway. Significantly, both PDIs are *N*-glycosylated, and at least one possesses extensive *N*-glycans containing poly-*N*-acetyllactosamine, which indicates post-translational modification of the protein in the Golgi complex.

EXPERIMENTAL PROCEDURES

Trypanosomes—Procyclic *T. brucei* EATRO1125 cells were grown at 27 °C in SDM-79 medium supplemented with 15% fetal bovine serum (13). Bloodstream forms of *T. brucei* were grown at 37 °C in HMI-9 medium supplemented with 10% fetal bovine serum and 10% SERUM PLUS™ (14). Cell media were supplemented with G418 (2 μg/ml) for maintenance of the 328.114 cell line and with both G418 (2 μg/ml) and hygromycin (2 μg/ml) for maintenance of the 13.90 cell line. The *T. brucei* variant clone AnTat 1.1 was grown in mice, and trypanosomes were purified as described previously (15).

Cloning and Identification of PDI Genes—A cDNA expression library was constructed in λ-Zap using poly(A) mRNA isolated from bloodstream

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[1] The abbreviations used are: PDI, protein disulfide isomerase; Tb-PDI, *Trypanosoma brucei* protein disulfide isomerase; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone or N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione *S*-transferase; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulfonic acid; RNAi, RNA interference.

forms of T. *brucei* (AnTat 1.1). Polyclonal antibodies prepared against a fraction of *T. brucei* proteins that bind specifically to tomato lectin were used to screen 2.5×10^5 clones. The positive plaques were purified by repeated screening. The isolated cDNA inserts were excised out of the pico-Bluescript-cytomegalovirus phagemid vector. The resulting phagemids were added to *Escherichia coli* XLOR bacteria as part of the excision process. Sequencing of the DNA was performed using the Amersham Biosciences thermo sequenase radiolabeled terminator cycle sequencing kit. Sequence analysis was carried out using the European Molecular Biology Laboratory (EMBL) Parasite Genomes WU-Blast2 program.

Protein Induction and Antibody Production—Fragments of 1.2 and 1.5 kb corresponding to the open reading frames of the *TbPDI-1* and *TbPDI-2* genes, respectively, were amplified by PCR. The forward and reverse primers for *TbPDI-1* contained EcoRI and NotI sites and were 5′-TCGAATTCTCCTCTGGTGTTGTGGAGCTA-3′ and 5′-ATGCGGCCGCTCCTTCGGCTGCTCCTTCGT-3′, respectively; whereas the forward and reverse primers for *TbPDI-2* contained BamHI and XhoI sites and were 5′-TCCGGATCCCCCATGCGCGCTATTTTTCTTGTTGC-3′ and 5′-GAGCTCGAGCTACAGATCTTGTTTATCAACATTG-3′, respectively (corresponding restriction sites are underlined). Digestion with these restriction enzymes allowed the insertion of the amplification product into the pGEX-5x-1 multiple cloning site. Flanking primers specific for the pGEX vector were employed to sequence the constructs and confirm that both open reading frames had been cloned in-frame with the GST open reading frame of the expression vector. Expression of the recombinant fusion proteins in *E. coli* (DH10B) was induced by an overnight incubation with 1 mM isopropyl-β-D-1-thiogalactosidase with gentle agitation at 15 °C. Bacteria were harvested by centrifugation ($4000 \times g$, 15 min); resuspended in ice-cold phosphate-buffered saline (PBS; pH 7.3) containing 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl, 30 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM antipain, 20 μM E-64, and 50 μM TLCK; and lysed by sonication on ice. Soluble proteins were separated from the insoluble proteins by retrieval of the supernatant after centrifugation ($4000 \times g$, 15 min). The recombinant GST fusion proteins were purified by affinity chromatography using glutathione-Sepharose (Amersham Biosciences). The purity of recombinant proteins was confirmed by SDS-PAGE and Coomassie Blue staining. Polyclonal anti-GST-PDI antibodies were raised against the purified recombinant fusion proteins in rabbits and mice as described previously (16).

Gels and Immunoblotting—SDS-PAGE and Western blots were performed as described previously (16).

DNA and RNA Analysis—The procedures for the isolation of DNA and RNA as well as Southern and Northern blot hybridizations have been described elsewhere (17).

N-Glycopeptidase F and Endoglycosidase H Digestion of Immunoprecipitates—The PDIs were immunoprecipitated from detergent lysates prepared by the method of Anderson and Blobel (18). The immune complexes were eluted from the protein A resin by boiling (2 min) in a phosphate buffer (pH 7.5) containing 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.1 M NaCl, and 4% (w/v) SDS followed by centrifugation ($9000 \times g$, 3 min). Four volumes of PBS containing 2.5% (w/v) Triton X-100 and 5 mM dithiothreitol were added to the denatured immunoprecipitated protein complexes, which were incubated on ice for 45 min. After centrifugation ($10{,}000 \times g$, 10 min), the supernatants were removed, and 0.2 volume of 0.25 M Na_2HPO_4 (pH 7.6) containing 50 mM EDTA and 5 mM dithiothreitol was added. The samples were incubated overnight at 37 °C in the presence and absence of *N*-glycopeptidase F (5 units/ml). The reaction was terminated by the addition of 5 μg of RNase A followed by 5 volumes of ice-cold acetone. After a 2-h incubation at −20 °C, samples were centrifuged ($9000 \times g$, 2 min), and the resulting pellets were air-dried. SDS-PAGE sample buffer was added to the samples, which were boiled (5 min) and subjected to electrophoresis. Digestion with endoglycosidase H (3 units/ml) was carried out exactly as described for *N*-glycopeptidase F digestion but in 50 mM $NaCH_3COO$ (pH 5.5) containing 5 mM dithiothreitol and 2.5 mM phenylmethylsulfonyl fluoride.

Tomato Lectin Blotting—Blots were incubated overnight at 4 °C with PBS containing 4 μg/ml biotinylated tomato lectin, 0.1 mM $CaCl_2$, and 2% (w/v) polyvinyl pyrrolinone, followed by three washes in 50 mM Tris (pH 7.5) containing 15 mM KCl and 5 mM $MgCl_2$. The blots were incubated at 4 °C for 3 h with streptavidin alkaline phosphatase (Promega) in 50 mM Tris (pH 7.5) containing 15 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1% (w/v) Tween 20 and then subjected to three washing steps (5 min) using PBS containing 0.1 mM $CaCl_2$ and 0.1% (w/v) Tween 20. The blots were then incubated in alkaline phosphatase developing solution containing 100 mM Tris (pH 9), 150 mM NaCl, and 1 mM $MgCl_2$ and the two substrates nitro blue tetrazolium and 5-bromo-1-chloro-3-indylo phosphate.

Preparation of Whole Cell Extracts—Bloodstream and procyclic cells were washed once in PBS buffer containing 83 mM glucose and 128 mM sucrose and then resuspended in the same buffer before the addition of an equal volume of SDS loading buffer (2×) to give a concentration of 5×10^5 cells/μl. Samples were boiled immediately for 5 min.

Fractionation of Cells—For detergent extraction, bloodstream trypanosomes were incubated (1×10^9 cells/ml) on ice for 1 h in 50 mM Tes buffer (pH 7.5) containing 150 mM NaCl, 2% (w/v) CHAPS, 1 mM EDTA, 30 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM antipain, 20 μM E-64, and 50 μM TLCK. The extract was centrifuged ($12{,}000 \times g$, 30 min). The supernatant (the soluble fraction) was retained, and the pellet (the insoluble fraction) was washed once in 50 mM Tes (pH 7.5) containing 150 mM NaCl and then resuspended in 8 M urea. Bloodstream trypanosomes were also fractionated by mechanical disruption using a combination of sonication and a freeze-thaw cycle. The cells were suspended in 25 mM Tris buffer (pH 7.5) containing 20 mM NaCl, 1 mM EDTA, 30 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM antipain, 20 μM E-64, and 50 μM TLCK and subjected to a 30-s sonication step followed by a single freeze-thaw cycle. The soluble fraction was isolated by centrifugation ($12{,}000 \times g$, 30 min), and the insoluble or pellet fraction was treated as described above for detergent extraction.

Enzymatic Assays—Purified recombinant GST-PDI was used in each assay. The ability of PDIs to catalyze the refolding of "scrambled" bovine pancreatic RNase type III-A was determined as described by Freedman and co-workers (19–21). The ability of PDIs to reduce the disulfide bonds between insulin chains with 0.5 mM dithiothreitol was measured as the change in absorbance at 650 nm (20).

Immunofluorescence and Confocal Microscopy—Bloodstream trypanosomes (AnTat1.1; 1×10^7 cells/ml) were fixed in PBS containing 3% (w/v) paraformaldehyde on ice for 10 min. The fixed cells were washed once in PBS containing 5% fetal bovine serum and 0.01% (w/v) Tween 20 and resuspended in the same solution (2.5×10^7 cells/ml). The cell solution was smeared onto poly-L-lysine-coated glass slides and air-dried. Slides were incubated in acetone and then incubated in methanol at −20 °C for 5 min each. After blocking nonspecific protein binding sites with the PBS/fetal bovine serum/Tween 20 solution, the cells were treated with primary antibody solutions followed by the appropriate secondary antibodies (anti-mouse IgG/IgM or anti-rabbit IgG) conjugated to fluorescein isothiocyanate, Alexa 594, or Texas Red. The anti-CB1 mouse monoclonal IgM was obtained from Cedarlane. The slides were then washed with PBS/fetal bovine serum/Tween 20 solution before mounting in PBS containing 50% (w/v) glycerol and 4′,6-diamidino-2-phenylindole (DAPI) blue stain (0.1 μg/ml). Images were taken on a Zeiss Axioskop 2 microscope coupled to a charge-coupled device camera and processed by ISIS 3. Confocal microscopy was carried out using a Lecia TCS SP2 confocal microscope.

Generation of RNAi Constructs—A 500-bp fragment from the open reading frame of each *TbPDI* gene (nucleotides 280–851 of *TbPDI-1* cDNA and nucleotides 537–1076 of *TbPDI-2* cDNA) was amplified by PCR using primers that incorporated HindIII and XhoI sites at the respective 5′- and 3′-ends of the amplification products. These products were ligated into the pZJM vector using the same restriction sites. The pZJM vector carries a phleomycin resistance gene and upon transfection was integrated into the ribosomal DNA spacer region of the genome (22). The p2T7ver3-PDI-2 construct was generated by subcloning the 500-bp amplification product of the *TbPDI-2* gene directly into vector that had been previously digested with XcmI (23). The 500-bp fragment of the *PDI* gene was amplified by PCR using *Thermus aquaticus* (*Taq*) polymerase (Sigma), which added a single adenosine to the 3′-ends of the double-stranded DNA product. The ligation was mediated by the complementarity between the PCR product 3′-A overhang and the vector 3′-T overhang generated by digestion of XcmI. The p2T7ver3 vector carries a hygromycin resistance gene and is integrated into the 177-bp repeats of the mini-chromosomes (23).

RNA Interference of TbPDI Genes—The bloodstream form cell line 90-13 contains integrated genes for both T7 polymerase (pLEW 13/G418 selection) and the tetracycline repressor (pLEW90/hygromycin selection) and requires both antibiotics for maintenance (22). Transfection of these cells (400 μl of 2×10^7 cells) with 20 μg of NotI-linearized DNA was carried out as described previously (22). Transfected cells were incubated for 6–8 h in HMI-9 medium at 37 °C before drug selection (2.5 μg/ml phleomycin) in 24-well plates. RNA interference was induced by incubation with tetracycline (2 μg/ml). The single marker bloodstream *T. brucei* cell line (328.114) described by Wirtz *et al.* (24, 25) contains both T7 polymerase and tetracycline repressor genes on the same cassette and requires only one selection drug (G418). Cells were transfected with the NotI-linearized pZJM-PDI-1 construct exactly as

described above. After drug selection, the same cells were re-transfected with a NotI-linearized p2T7ver3-PDI-2 construct to obtain a double RNAi cell line after selection with phleomycin and hygromycin.

RESULTS

Isolation and Characterization of Two PDI Genes from T. brucei—Polyclonal antibodies were raised against proteins present in the tomato lectin binding fraction isolated from bloodstream forms of *T. brucei*. These antibodies were affinity-purified and used to screen a *T. brucei* bloodstream form cDNA expression library. A total of 2.5×10^5 clones were screened, and more than 100 positive plaques were isolated and subjected to three additional rounds of screening to generate a collection or "mini-library" of cDNAs that potentially coded from proteins present in the endocytic pathway/flagellar pocket. Sequence analysis revealed that these cDNAs fell into three groups. First, there were cDNAs coding for known resident proteins of the flagellar pocket/endocytic pathway such as CB1/P67 and EARLRAEE (26, 27). A second group of cDNAs (full length and partial) had no significant homology with proteins in current databases. The last group represented potential homologues of known proteins not previously reported to be associated with the endocytic pathway. Two cDNAs of the latter group were especially prominent, and based on their similarity with other sequences, these cDNAs were predicted to code for protein disulfide isomerases (Fig. 1). These potential homologues were termed TbPDI-1 and -2 because they respectively possessed one or two thioredoxin-like domains containing the double-cysteine redox-active C*XX*C motif (Fig. 1). TbPDI-1 was encoded by a cDNA (~1.7 kb) that contained a single open reading frame for a polypeptide of 413 residues with a predicted molecular mass of 45 kDa and pI of 9.0. This protein contained a typical PDI redox-active site (FYAPWCGHCK) located between residues 54 and 63 as well as an N-terminal signal peptide (Fig. 1*B*). Thus, TbPDI-1 appeared to be a member of the recently described group of PDIs found in other protozoan parasites that contain a single redox-active site (28–30). Interestingly, the protein did not possess the C-terminal KDEL-type endoplasmic reticulum retention motif found on most PDIs. A search of the parasite gene databases revealed that the highest identity (75%) was with a single domain PDI in the genome data base of the African trypanosome *T. congolense.* A lower level of identity was observed with sequences in the more distantly related parasites *T. cruzi* and *Leishmania major*, which respectively exhibited 54% and 40% identity with TbPDI-1 (Fig. 1*B*). TbPDI-2 was encoded by a full-length cDNA (~2 kb) that coded for a polypeptide of 497 residues with a predicted molecular mass of 55 kDa and a pI of 5.3 and was identical to the *BS2* gene product described by Hsu *et al.* (9). TbPDI-2 contained two double-cysteine, redox-active sites located at the C- and N-terminal regions of the protein and separated by 224 residues (Fig. 1*C*). Therefore, TbPDI-2 corresponded to a class 1 PDI according to the classification of Kanai *et al.* (31). As observed for most PDIs described so far, TbPDI-2 has an N-terminal signal sequence and a C-terminal KDEL-like endoplasmic reticulum retention motif. The highest identity was with a sequence identified in the *T. congolense* data base (65% identity). The level of identity was lower with PDI sequences from less closely related parasitic protozoans such as *T. cruzi*, *L. major*, and *Plasmodium falciparum*, which respectively had 50%, 36%, and 30% identity with TbPDI-2, whereas identity with metazoan class 1 PDIs was typically less than 25%. A closer analysis of the sequence of TbPDI-2 revealed two features apparently unique to the class 1 PDIs from African trypanosomes. First, the C-terminal redox-active site (PWCGHCK) was identical to the classical motif found in most PDIs, but the N-terminal redox-active site (VDTCGYCQ) in TbPDI-2 was atypical. Second, TbPDI-2 contained multiple consensus sites for *N*-glycosylation (a total of 12), with an obvious clustering of five sites in a unique stretch of 26 residues (residues 465–490) located between the C-terminal redox-active domain and the potential KDEL retention sequence. Interestingly, the PDI-2 homologues from *T. congolense* and *T. vivax* also have atypical N-terminal redox centers and possess multiple *N*-glycosylation sites, *e.g.* 15 in the case of *T. vivax*. However, there was only a single *N*-glycosylation site in the homologue from *P. falciparum*, and none at all in the case of *T. cruzi, L. major*, and metazoan organisms. In addition, in all of these parasites, the C-terminal redox centers were of the standard type. This analysis suggested that African trypanosomes possess an unusual class 1 PDI.

PDI Genes in Parasitic Protozoans—The isolation of two genes for PDIs from *T. brucei* prompted us to search the almost complete parasite genome data base for potential genes for *PDIs* in *T. brucei*, *T cruzi*, and *L. major* (Table I). The genome of each of these parasites contained at least five genes that were predicted to code for PDIs based on level of sequence identity, size, and the presence of thioredoxin-like redox-active sites, N-terminal signal peptides, and C-terminal endoplasmic reticulum retention sequences. In the case of each parasite, three of these PDIs were single domain PDIs and can be arranged into a hierarchy of small, medium, and large proteins, based on their expected size (ranging from 15 to 65 kDa). In addition, all three parasites contained two genes that code for PDIs possessing the classical arrangement of two thioredoxin-like domains containing the double-cysteine redox-active C*XX*C motif. Based on the location of these two domains, these genes can be subdivided into a single representative of a class 1 and 2 PDI (31). The analysis also supported the view that *T. brucei* contains an unusual class 1 PDI (TbPDI-2) in terms of the number of potential glycosylation sites and the atypical redox site. These features were not present in any other PDI from *T. brucei* or the other parasite genomes.

Enzymatic Activities of Recombinant TbPDI-1 and TbPDI-2—In order to determine whether TbPDI-1 and TbPDI-2 possessed activities associated with PDIs, recombinant proteins were expressed in *E. coli* as GST fusion products. Upon induction, both fusion proteins were recovered in the soluble fraction isolated after disruption of the bacteria and affinity-purified using immobilized glutathione. The ability to refold scrambled RNase is the standard assay for PDI activity, and recombinant TbPDI-1 and TbPDI-2 both had significant activity in catalyzing the restoration of RNase A activity in this assay (Fig. 2*A*). Recombinant TbPDI-1, but not TbPDI-2, was also able to catalyze the reduction of insulin in the standard reduction assay (Fig. 2*B*). Both of these activities have also been observed with a similar-sized single domain PDI from *Giardia lamblia* and *L. donovani* (28, 30).

Genomic Organization and Expression of TbPDI-1 and TbPDI-2—Analysis of the genome of *T. brucei* predicted that that both genes are present as single copies, and this status was confirmed directly by Southern blotting (data not shown). Northern blot analysis indicated that the transcripts for both genes were significantly more abundant in bloodstream than procyclic forms of the parasite (Fig. 3*A*). These results were in agreement with those of Hsu *et al.* (9), who observed a 15-fold down-regulation of the *BS2* mRNA in procyclic forms. Western blot analysis, using polyclonal antibodies raised against the recombinant proteins, demonstrated directly that both TbPDI-1 and TbPDI-2 were expressed only in bloodstream forms (Fig. 3*B*). Antibodies against TbPDI-1 did not cross-react with TbPDI-2 and vice versa, which was consistent with the low overall identity between the two proteins. Significantly, this analysis also demonstrated that TbPDI-2 migrated with an

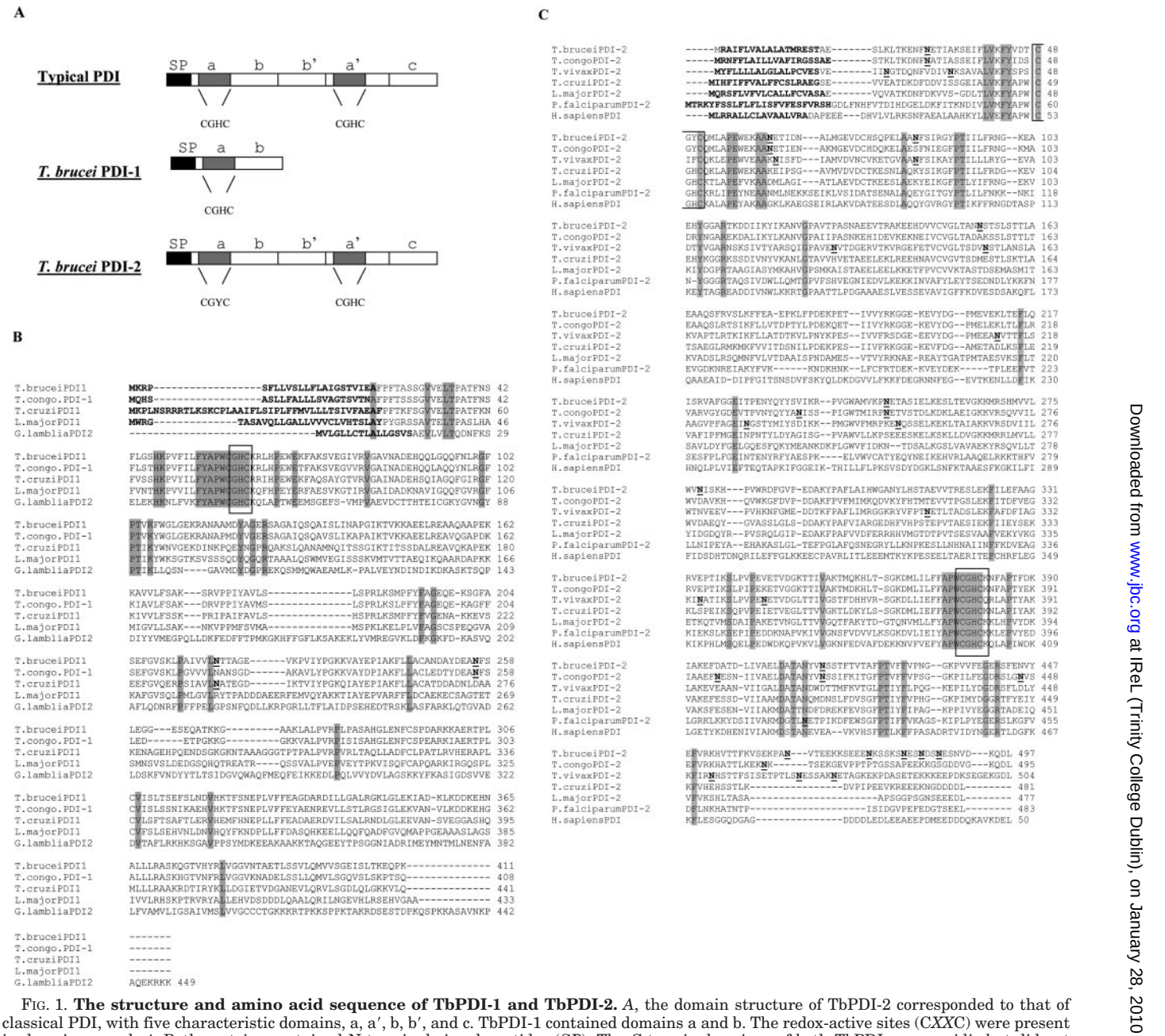

FIG. 1. **The structure and amino acid sequence of TbPDI-1 and TbPDI-2.** *A*, the domain structure of TbPDI-2 corresponded to that of classical PDI, with five characteristic domains, a, a′, b, b′, and c. TbPDI-1 contained domains a and b. The redox-active sites (C*XX*C) were present in domains a and a′. Both proteins contained N-terminal signal peptides (*SP*). The C-terminal regions of both TbPDIs were acidic but did not contain the classical (KDEL) endoplasmic reticulum retention signal. *B*, multiple sequence alignment of *T. brucei* PDI-1 (Sanger sequencing project Tb04.1H19.870) with *G. lamblia* PDI-2 (GenBank™ accession number U65017) and homologues from *T. cruzi* (Sanger sequencing project Tc00.1047053506559.200), *L. major* (Sanger sequencing project LmjF34.2200), and *T. congolense* (sequence assembled manually using data from the Sanger pathogen sequencing unit and OMNIBLAST searches). *C*, multiple sequence alignment of *T. brucei* PDI-2 (GenBank™ accession number JO2865) with PDI from *L. major* (GenBank™ accession number AAN75008), *Homo sapiens* (GenBank™ accession number Z49835), *P. falciparum* (GenBank™ accession number AJ250363) and homologues from *T. cruzi* (Sanger sequencing project Tc00.1047053507611.370) and *T. vivax* and *T. congolense* (both sequences assembled manually using data from the Sanger pathogen sequencing unit and OMNIBLAST searches). Amino acids identical in all sequences are shaded in *gray*, putative N-terminal signal peptides are in *bold*, conserved thioredoxin sites are *boxed*, and predicted *N*-glycosylation sites are *underlined*.

apparent molecular mass of ~80 kDa, which was significantly higher than the 55-kDa mass predicted by the primary sequence of the polypeptide. In contrast, TbPDI-1 migrated close to the expected size of 45 kDa.

Characterization of TbPDI-1 and TbPDI-2—Bloodstream forms of *T. brucei* were subjected to detergent extraction or mechanical disruption and separated into pellet and supernatant fractions by centrifugation. Western blot analysis of these fractions demonstrated that both PDIs were recovered to same extent in the supernatant fraction after detergent extraction or mechanical disruption, which indicated that TbPDI-1 and TbPDI-2 behave as soluble proteins (Fig. 4*A*). As expected, both proteins were detected along with ISG_{100} (8) in the tomato lectin binding fraction isolated from *T. brucei*, whereas tubulin was not (Fig. 4*B*). The large discrepancy between the observed and expected size of TbPDI-2 as well as the presence of multiple *N*-glycosylation sites raised the possibility that the protein was modified by *N*-glycosylation, a feature observed for several proteins that bind to tomato lectin (8). In order to investigate *N*-glycosylation of these TbPDIs, the proteins were immunoprecipitated from detergent extracts of cells using rabbit or mouse antibodies and then digested with *N*-glycopetidase F

TABLE I
Comparison of putative PDIs from parasitic protozoans

The sequences were identified by OMNIBLAST analysis of parasite genome databases (Gene DB) of *T. brucei*, *T. cruzi*, and *L. major*. The table presents the predicted size, the sequence of the active sites, and a classification of putative PDIs according to Kanai *et al.* (31).

	Predicted size	*N*-Glycosylation sites	Redox-active motif	Class	Leader sequence
	kDa				
T. brucei					
Tb10.6k15.2290 (PDI-2)	55	12	CGYC/CGHC	Class 1	Yes
Tb07.27M11.560	42	0	CGHC/CGHC	Class 2	Yes
Tb07.10C21.170	15	0	CGHC	Single domain	Yes
Tb04.1H19.870 (PDI-1)	45	2	CGHC	Single domain	Yes
TB05.28F8.290	65	0	CGHC	Single domain	Yes
T. cruzi					
Tc00.1047053507611.370	53	0	CGHC/CGHC	Class 1	Yes
Tc00.1047053508209.140	42	0	CGHC/CGHC	Class 2	Yes
Tc00.1047053506559.200	48	1	CGHC	Single domain	Yes
Tc00.1047053508173.150	26	0	CVHC	Single domain	Yes
Tc00.1047053472777.30	18	0	CGHC	Single domain	Yes
L. major					
LmjF36.6940	52	0	CGHC/CGHC	Class 1	Yes
LmjF26.0660	40	0	CGHC/CGHC	Class 2	Yes
LmjF34.2200	47	0	CGHC	Single domain	Yes
LmjF06.1050	15	0	CGHC	Single domain	Yes
LmjF26.0670	19	1	CRYC	Single domain	Yes

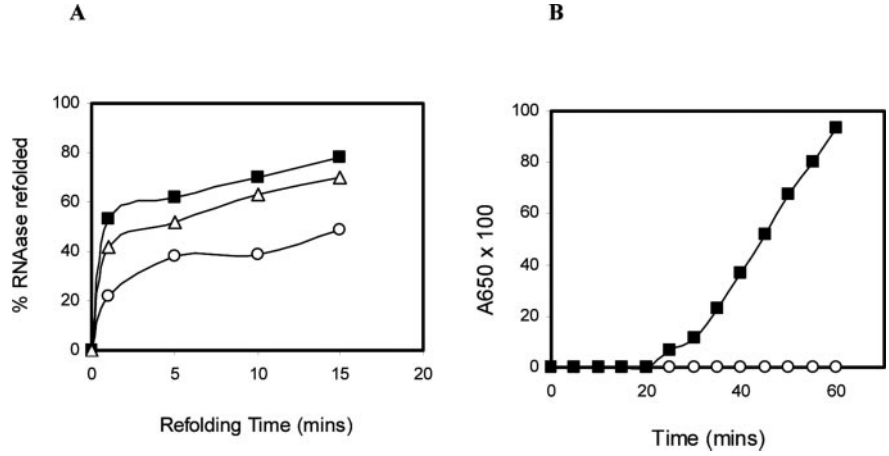

FIG. 2. **Assay of TbPDI-1 and TbPDI-2.** *A*, the ability of TbPDIs (0.5 μg) to restore enzymatic activity to scrambled RNase was determined by monitoring the activity of the refolded RNase as described under "Experimental Procedures." ■, TbPDI-1; △, TbPDI-2; ○, buffer control minus TbPDIs. *B*, the TbPDI-catalyzed reduction of insulin was measured spectrophotometrically at 650 nm. In each case, the data are from a typical representative of multiple assays performed as described under "Experimental Procedures." ■, TbPDI-1; ○, TbPDI-2.

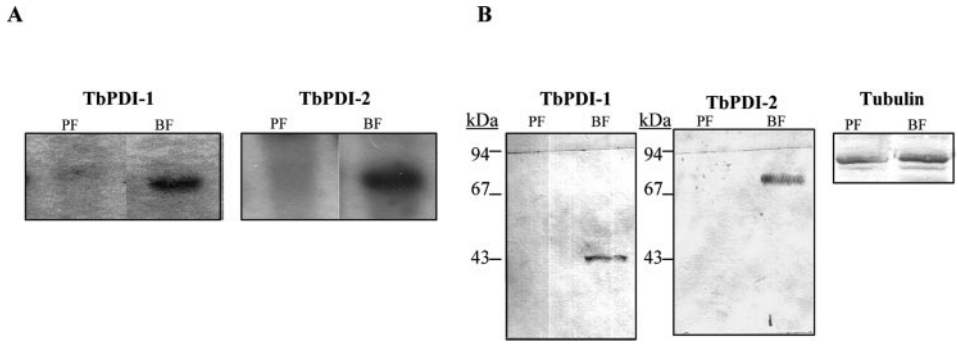

FIG. 3. **Expression of the genes for TbPDI-1 and TbPDI-2.** *A*, total RNA (15 μg) from bloodstream form (AnTat 1.3A) and procyclic form (EATRO 1125) trypanosomes was separated on a formaldehyde agarose gel, transferred to Hybond-C extra nitrocellulose filters, and probed with a ^{32}P-labeled EcoRI and NotI fragment of the *TbPDI-1* gene and a ^{32}P-labeled EcoRI and XbaI fragment of the *TbPDI-2* gene. *B*, Western blots of whole cell extracts from procyclic and bloodstream form trypanosomes (5×10^6 cells) were probed with antibodies against the recombinant PDIs. The same extracts were probed with anti-tubulin antibodies as a loading control.

(which cleaves most *N*-glycans) or endoglycosidase H (which cleaves high mannose or hybrid but not complex structures). These digests were then subjected to Western blot analysis using the corresponding rabbit or mouse anti-PDI antibodies (Fig. 4, *C* and *D*). The results clearly demonstrated that incubation of TbPDI-2 with *N*-glycopeptidase F resulted in a significant shift in the apparent molecular mass of the protein, which now migrated close to the expected size of 55 kDa. However, treatment with endoglycosidase H only resulted in a small shift (~5 kDa) in the apparent molecular mass of the protein (Fig. 4*D*). In contrast, treatment of PDI-1 with *N*-glycopeptidase F or endoglycosidase H resulted in the same small shift in apparent molecular mass (Fig. 4, *C* and D). Taken together, these results support the view that TbPDI-2 is extensively *N*-glycosylated and that the majority of these glycans are of the complex type and contain poly-*N*-acetyllactosamine. In contrast, PDI-1 possesses only relatively short *N*-glycan chains, and these are unlikely to be complex in nature. This view was

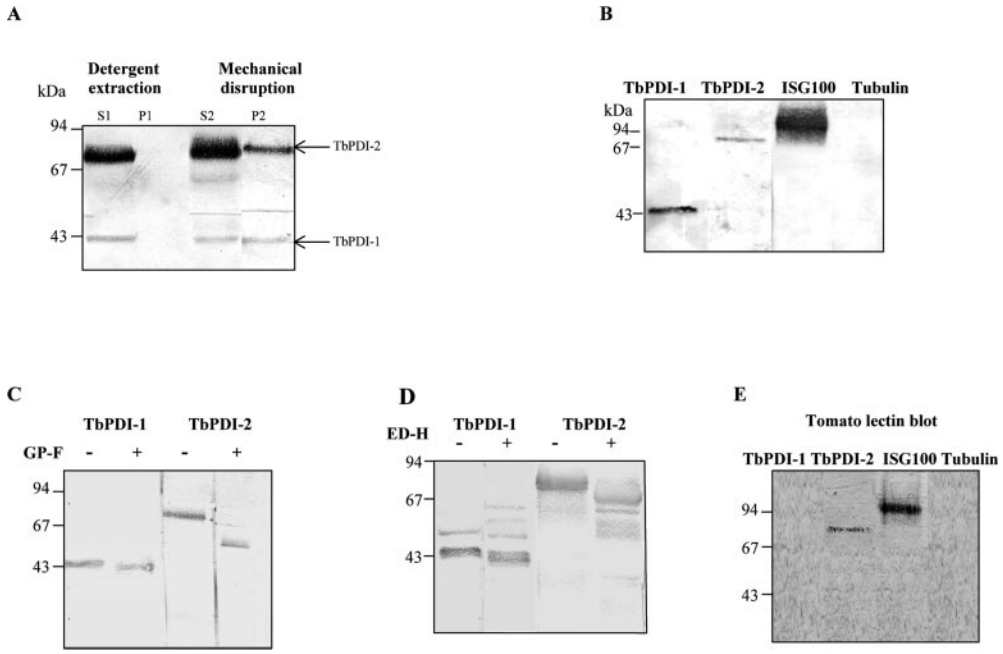

FIG. 4. **Characterization of TbPDI-1 and TbPDI-2.** *A*, bloodstream trypanosomes were fractionated by detergent extraction into a soluble supernatant fraction (*S1*) and a detergent-insoluble pellet fraction (*P1*). Proteins were also fractionated by mechanical disruption, resulting in a soluble supernatant fraction (*S2*) and an insoluble pellet fraction (*P2*). All fractions were probed with the mouse antibodies against both TbPDIs. *B*, the tomato lectin binding fraction isolated from bloodstream trypanosomes was probed on a Western blot using antibodies against both TbPDIs, ISG_{100}, and tubulin, which served as a negative control. *C*, *N*-glycopeptidase F digestion of TbPDIs. The TbPDIs were immunoprecipitated using the appropriate mouse serum. The immunoprecipitated TbPDIs were mock-treated (*lanes 1* and *3*) or treated (*lanes 2* and *4*) with *N*-glycopeptidase F (*GP-F*). Samples were analyzed on an SDS-PAGE gel, and blots were subsequently probed with the rabbit anti-TbPDI antibodies. *D*, endoglycosidase H digestion. The TbPDIs were immunoprecipitated from cells using rabbit antibodies and then probed with the mouse antibodies after treatment with endoglycosidase H (*ED-H*). The immunoprecipitated TbPDIs were mock-treated (*lanes 1* and *3*) or treated (*lanes 2* and *4*) with endoglycosidase H. *E*, tomato lectin blot. The TbPDIs, ISG_{100}, and tubulin were immunoprecipitated from bloodstream trypanosomes using appropriate rabbit serum. Immunoprecipitates were analyzed by SDS-PAGE and tomato lectin blotting. The position of molecular mass standards is indicated to the *left* of each panel.

supported by the results of tomato lectin blots, which demonstrated directly that TbPDI-2 and ISG_{100} but not TbPDI-1 and tubulin interact with tomato lectin (Fig. 4*E*).

Localization of TbPDI-1 and TbPDI-2—The localization of TbPDI-1 and TbPDI-2 in fixed cells was investigated by indirect immunofluorescent antibody staining of fixed cells (Fig. 5*A*). Both proteins were located primarily in the posterior end of the cell and clearly concentrated between the nucleus and kinetoplast. This region of the cell is the site of the highly active endocytic pathway in bloodstream trypanosomes. Significantly, there was good co-localization of both TbPDIs with p67/CB-1, a resident membrane glycoprotein of the lysosome and endocytic pathway. (Fig. 5*B*) (32–34). Confocal microscopy was employed to investigate in more detail the localization of both TbPDIs (Fig. 5*C*). These high resolution immunolocalizations were performed in parallel with co-localization studies with tomato lectin, which is an established marker for the endocytic pathway of *T. brucei* (8). There was a strong correlation between the localization of TbPDI-1 and TbPDI-2 and fluorescein isothiocyanate-labeled tomato lectin in a series of sections taken through the same cells (Fig. 5*C*). Taken together, these data from epifluorescence and confocal microscopy supported the view that TbPDI-1 and TbPDI-2 were resident enzymes of the endocytic pathway in bloodstream forms of *T. brucei.*

TbPDI-1 and TbPDI-2 Are Not Essential—Conditional ablation of mRNAs through RNA interference has become a powerful method for investigating gene function in trypanosomes (35–40). We employed a construct that allowed the tetracycline-inducible production of a double-stranded RNA containing 500 bp of the open reading frame of *TbPDI-1* or *TbPDI-2*. Analysis of growth curves over a period of 15 days indicated that the presence of tetracycline had no effect on the growth of the TbPDI-1 and -2 RNAi cells, although these cell lines grew at slightly lower rate than observed for the parental cell line used for the RNAi analysis (Fig. 6*A*). Significantly, the mRNA for *TbPDI-2* was barely detectable, whereas the *TbPDI-1* transcript was absent after a 48-h induction of the double-stranded RNA with tetracycline (Fig. 6*B*). In addition, both TbPDIs were no longer detectable by Western blot analysis after a 48-h induction of the corresponding double-stranded RNA, and this loss was maintained up to 1 week of induction (Fig. 6*C*). A double RNAi cell line was generated to determine whether the loss of both TbPDIs affected growth. Significantly, induction of the double-stranded RNA for *TbPDI-1* and *TbPDI-2* had no effect on the growth of the double RNAi cells cells, even though a Western blot was consistent the complete loss of both proteins in cells incubated in the presence of tetracycline (Fig. 6*D*). Taken together, these results indicated that neither TbPDI, either individually or in combination, was essential for growth of bloodstream forms, at least in culture.

DISCUSSION

PDIs are well-described members of the thioredoxin superfamily that are considered to be essential in eukaryotes because of their role in ensuring the formation of correct disulfide bonds and the subsequent processing and maturation of proteins in the endoplasmic reticulum (10–12). This study presents the first detailed characterization of two PDIs from the African trypanosome *T. brucei*. These TbPDIs possess several unusual features that distinguish them from described previously PDIs. First, the expression of TbPDI-1 and -2 is clearly developmentally regulated at the mRNA and protein level and is restricted to bloodstream forms of the parasite. Neither enzyme is expressed in procyclic forms of *T. brucei*, which are

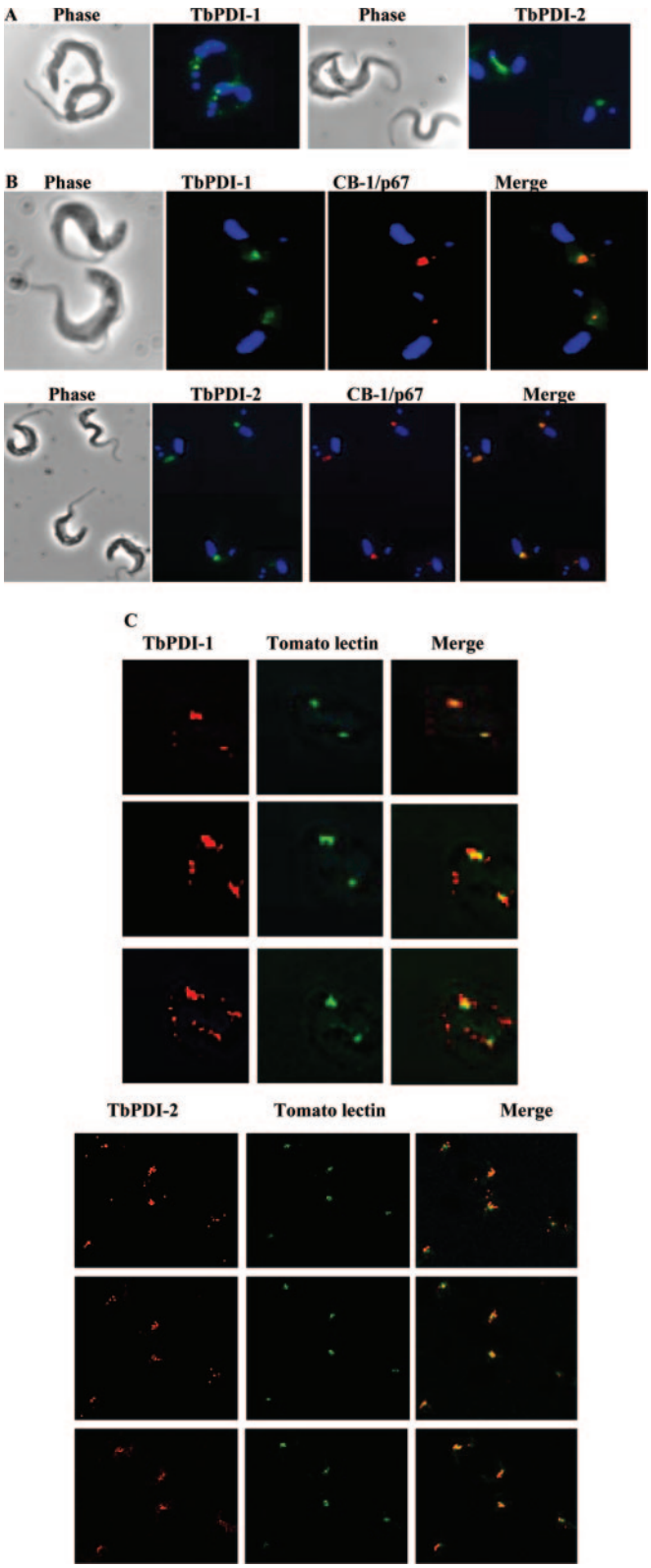

FIG. 5. **Localization of TbPDI-1 and TbPDI-2.** Bloodstream trypanosomes were fixed and probed with anti-TbPDI mouse serum. 4′,6-Diamidino-2-phenylindole stain (*blue*) indicated the position of nucleus and kinetoplast. *A*, localization of TbPDIs. *B*, co-localization of PDI (*green*) and CB-1/p67 (*red*). *C*, confocal microscopy demonstrating co-localization of TbPDIs (*red*) and tomato lectin binding (*green*) in a series of sections through fixed cells.

the major proliferative stage in the tsetse fly vector. In contrast, the expression of PDIs in other parasitic protozoans, *e.g. G. lamblia* (28–29) or *L. donovani* (30), does not appear to vary during the life cycle. An interesting exception might be the single class 1 PDI from *L. major*, which is more abundant in highly virulent strains of the parasite (41). However, to date, the expression of a PDI has not been reported to be restricted solely to a specific life cycle stage of any parasitic protozoan. The same appears to be true for metazoan organisms, in which expression of PDIs also appears to be constitutive, although the actual levels may be higher during fetal development, when large amounts of proteins are being synthesized (42). A pancreas-specific PDI family member appears to be the sole example of a tissue-specific form in adults (43). Thus, the finding of two developmentally regulated PDIs in *T. brucei* contrasts with the consensus view in the literature that PDIs are constitutively expressed in eukaryotic organisms because of their essential role in protein folding.

A second significant difference between the TbPDIs and other PDIs is that both proteins are post-translationally modified by *N*-glycosylation. It seems unlikely that glycosylation is required for enzymatic activity because both enzymes possess isomerase activity when expressed in *E. coli*. It is generally accepted that *N*-glycosylation is rare among enzymes, and to date, the only previous example of a glycosylated PDI is a class 1 PDI from yeast (44). The extent of glycosylation of TbPDI-1 appears to be modest and apparently contributes ~2–3 kDa to the mature form of the protein, which was also the case for the yeast PDI (44). These glycans can be completely removed with *N*-glycopeptidase F or endoglycosidase H and probably represent an endoplasmic reticulum glycoform or possibly a small hybrid structure. Although TbPDI-1 was detected in the isolated tomato lectin binding fraction, the protein is unlikely to contain poly-*N*-acetyllactosamine because it was not detectable by tomato lectin blotting. Therefore, the presence of TbPDI-1 in the tomato lectin binding fraction may be due to association with proteins that contain poly-*N*-acetyllactosamine (8). In contrast, *N*-glycans account for a significant proportion of the apparent molecular mass of native TbPDI-2. Moreover, the significant differential sensitivity of TbPDI-2 to *N*-glycopeptidase F and endoglycosidase H suggests that the majority of these glycans are likely to be complex rather than high mannose or hybrid structures. In addition, TbPDI-2 is a constituent of tomato lectin binding fraction and was also detectable by tomato lectin blotting. Together, these data indicated that TbPDI-2 is modified by the addition of glycans containing linear repeats of poly-*N*-acetyllactosamine and represents the first example of such a modification for any PDI. The biosynthesis of complex carbohydrate chains and the addition of poly-*N*-acetyllactosamine are thought to occur exclusively in the Golgi complex. Interestingly, neither TbPDI appears to be secreted because they were not detectable in the medium of cells growing in culture (data not shown). These considerations imply that a trafficking pathway must operate for TbPDI-2 that involves export of the newly synthesized protein from the endoplasmic reticulum followed by modification in the Golgi because only the mature fully glycosylated form of the protein was detected in cells.

There are several indications that class 1 PDIs from other African trypanosomes are processed in a similar fashion. First, the related proteins from *T. congolense* and *T. vivax* also contain an unusually high number of potential *N*-glycosylation sites. Second, a Western blot analysis revealed that antibodies against TbPDI-2 cross-reacted with a protein with a molecular mass of ~80 kDa in these trypanosomes (data not shown), which was significantly higher than the mass of 55 kDa predicted from the sequences in the parasite genome data base. Finally, tomato lectin also binds exclusively to proteins from

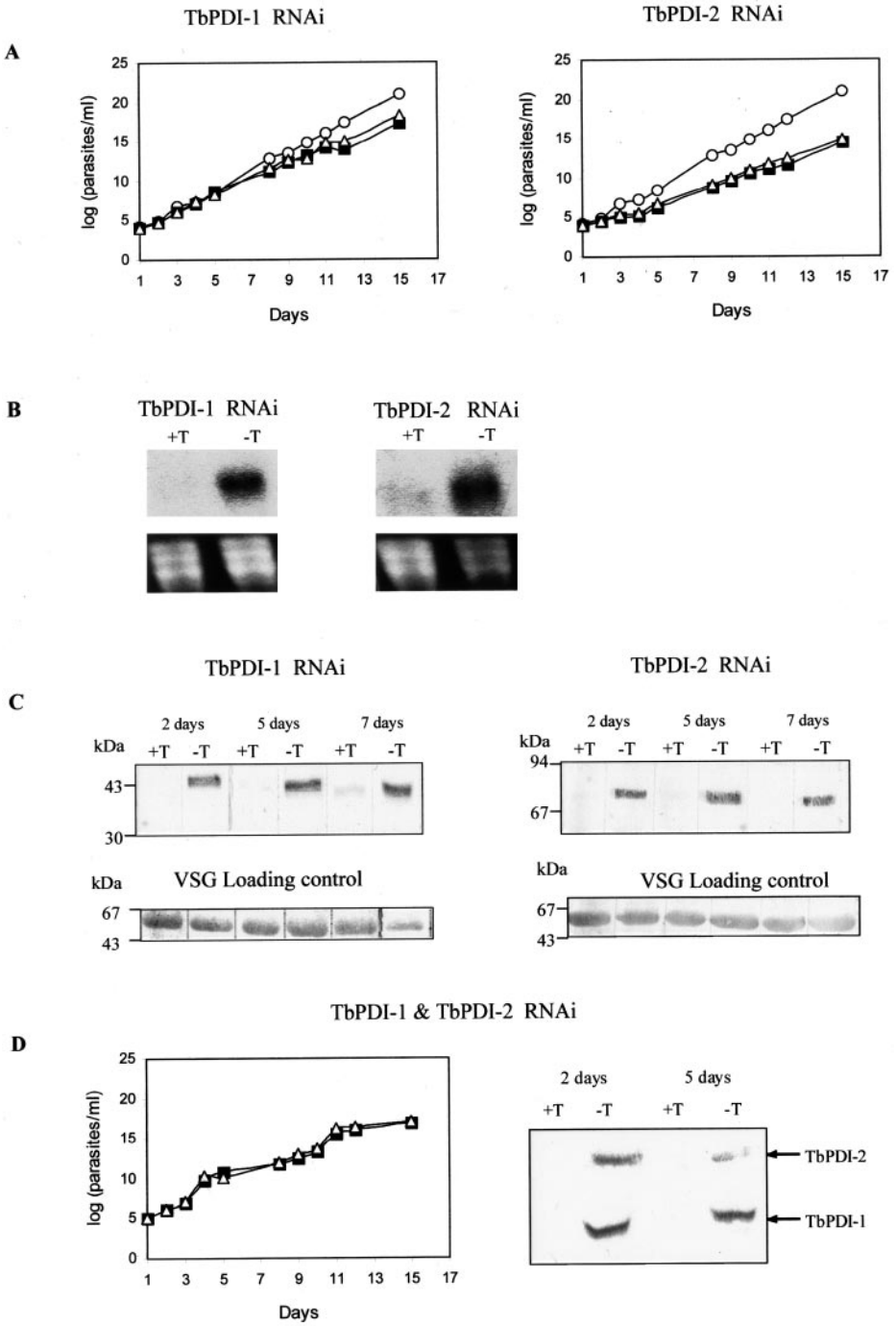

FIG. 6. **RNA interference of TbPDI mRNAs.** *A*, growth curves for TbPDI-1 and TbPDI-2 RNAi cells. ○, parental cell line 90-13; △, noninduced RNAi cells; ■, RNAi cells plus tetracycline. *B*, after a 2-day induction with tetracycline, total RNA (15 μg) was extracted from induced (+*T*) and non-induced cells (−*T*) for Northern blot analysis. *C*, whole cell lysates were prepared from induced (+*T*) and non-induced cells (−*T*) after 2-, 5-, and 7-day inductions; analyzed by SDS-PAGE gel; and blotted and probed with anti-PDI mouse sera. Each lane represents 2×10^6 cells. The same samples were probed with antibodies against the variable surface glycoprotein (*VSG*) as a loading control. *D*, the *left panel* presents the growth curves for the double RNAi (TbPDI-1 and TbPDI-2) cell line grown in the presence (■) and absence (△) of tetracycline. The *right panel* presents a Western blot analysis for TbPDI-1 and -2 in induced (+*T*) and non-induced (−*T*) double RNAi cells at various times. Each lane represents 2×10^6 cells.

the endocytic pathway in these trypanosomes.[2] All of these trypanosomal PDIs possess an unusual C-terminal redox-active center, (T/S)CG(Y/F)C. This motif is similar to the redox-active site (TCGYCH) of DsbC in *E. coli* (45). Interestingly, DsbC appears to act primarily as a reductant or isomerase in the bacterial periplasm, whereas another member of the family DsbA, which has a CPHC motif, acts as an oxidant to form disulfide bonds (46–48). Perhaps a similar role applies to the class 1 PDIs from African trypanosomes.

A third major difference between TbPDI-1 and -2 and PDIs in other eukaryotes is their subcellular location. The widely held view in the literature is that members of the PDI family are resident proteins of the lumen of the endoplasmic reticulum, where they were thought to play a key role in the folding and maturation of newly synthesized proteins (10–12). The evidence for secretion and other locations for PDIs has been attributed to leakage during isolation and cell fractionation or the propensity of PDIs to bind to unfolded or incorrectly folded proteins and peptides (12, 49). However, the localization studies presented here, involving standard epifluorescence and confocal sectioning, indicated that TbPDI-1 and TbPDI-2 co-localized with markers of the endocytic pathway. This localization was consistent with the novel *N*-glycosylation and trafficking of TbPDI-2, the presence of both proteins in the tomato lectin binding fraction, and the finding that neither protein is secreted. In other eukaryotes, lysosomal enzymes are routed to endosomes by a mannose 6-phosphate receptor that interacts in a reversible manner with phosphorylated mannose residues on *N*-glycans of the cargo protein (50). Interestingly, in a prescient comment, Hsu *et al.* (9) observed that the number of

[2] D. P. Nolan and N. B. Murphy, unpublished data.

N-glycosylation sites in BS2(TbPDI-2) was rare among enzymes and that if the protein was glycosylated, the location of the protein might be affected, even though a C-terminal KDEL-like sequence was present. However, there is no evidence for a phosphomannose trafficking mechanism in trypanosomes, nor do homologues of the mannose 6-phosphate receptor appear to be present in the genome. This leaves the possibility that the TbPDI glycans play a role in the trafficking of the protein from the Golgi to the endosomal compartments, as has been proposed for the poly-*N*-acetyllactosamine repeats of membrane proteins of the endocytic pathway in *T. brucei* (8). Alternatively, these glycans may function to protect the TbPDIs from the resident hydrolases present in the lumen of the endosomal/lysosomal compartments, as suggested for poly-*N*-acetyllactosamine-containing glycans of lysosomal membrane proteins in higher eukaryotes (51). Irrespective of how the TbPDIs are delivered to the endosomal compartments, their function in this location is likely to be different from the conventional oxidation functions ascribed to PDIs in the endoplasmic reticulum because endosomes are thought represent a more reducing environment (52).

Finally, the use of RNAi clearly indicated that these TbPDIs were not required for growth of bloodstream forms in culture. This was a surprising result, given that both proteins are expressed only in bloodstream forms and the general view that PDIs are essential proteins (10–12). The latter requirement has been demonstrated directly for the class 1 PDI from yeast, where the protein has an essential role in unscrambling non-native disulfide bonds (53, 54). Why do trypanosomes specifically up regulate the expression of these TbPDIs in bloodstream forms when they are apparently not required? It remains possible that residual levels of either PDI that are sufficient to support normal growth may remain in the RNAi cells, but the finding that growth was not affected in the double RNAi cells when neither TbPDI was detectable by Western blotting makes this unlikely. Another possibility is that there is redundancy of function, given that there are five potential TbPDIs in the genome. Although this possibility cannot be discounted unequivocally, it seems unlikely because the five putative TbPDIs are very different in class and size and have little sequence identity. A more intriguing possibility is that these TbPDIs are not required for growth *in vitro* but have a function *in vivo*. Interestingly, there is some evidence for an *in vitro* role for a class 1 PDI in *L. major*, where expression appears to be linked to virulence (41). What might this role be in the case of *T. brucei*? It is becoming clear that the high rates of endocytic activity and the clearance of surface-bound antibodies may be connected (55, 56). It has been proposed that the rapid clearance of surface-bound antibodies and their delivery to an endosomal/lysosomal compartment, where they are degraded to small fragments, form part of the parasite's defense against the host immune system and function to aid survival before the occurrence of a variable surface glycoprotein switching event (55). Given their location, expression profile, activity, and redundancy in culture, it is tempting to speculate that these TbPDIs might have a role in this mechanism, perhaps by assisting in the reduction of disulfide bridges or otherwise unfolding the internalized antibodies to allow for rapid proteolysis. This view is consistent with evidence in the literature that a PDI is responsible for the unfolding of internalized cholera toxin (57).

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