Intracellular Trafficking and Glycobiology of TbPDI2, a Stage-Specific Protein Disulfide Isomerase in *Trypanosoma brucei*

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*Trypanosoma brucei* protein disulfide isomerase 2 (TbPDI2) is a bloodstream stage-specific luminal endoplasmic reticulum (ER) glycoprotein. ER localization is dependent on the TbPDI2 C-terminal tetrapeptide (KQDL) and is mediated by TbERD2, an orthologue of the yeast ER retrieval receptor. Consistent with this function, TbERD2 localizes prominently to ER exit sites, and RNA interference (RNAi) knockdown results in specific secretion of a surrogate ER retention reporter, BiPN:KQDL. TbPDI2 is highly N-glycosylated and is reactive with tomato lectin, suggesting the presence of poly-N-acetyllactosamine modifications, which are common on lyso/endosomal proteins in trypanosomes but are inconsistent with ER localization. However, TbPDI2 is reactive with tomato lectin immediately following biosynthesis—far too rapidly for transport to the Golgi compartment, the site of poly-N-acetyllactosamine addition. TbPDI2 also fails to react with *Erythrina cristagalli* lectin, confirming the absence of terminal N-acetyllactosamine units. We propose that tomato lectin binds the Man₁-4GlcNAc₆1-4GlcNAc trisaccharide core of paucimannose glycans on both newly synthesized and mature TbPDI2. Consistent with this proposal, α-mannosidase treatment renders oligomannose N-glycans on the *T. brucei* cathepsin L orthologue TbCatL reactive with tomato lectin. These findings resolve contradictory evidence on the location and glycobiology of TbPDI2 and provide a cautionary note on the use of tomato lectin as a poly-N-acetyllactosamine-specific reagent.

African trypanosomes (*Trypanosoma brucei* spp.) are an ancient eukaryotic lineage of parasitic protozoa that cause human (sleeping sickness) and veterinary (nagana) disease throughout sub-Saharan Africa. Besides their direct impact as pathogens, trypanosomes and other closely related kinetoplastid parasites (*Trypanosoma cruzi* and *Leishmania*) have become important alternative model organisms for studying basic eukaryotic cell biology. In this regard trypanosomes have made significant contributions in two related areas: secretory trafficking and glycobiology. Most of this work has been done in the pathogenic bloodstream (BSF) and nonpathogenic insect procyclic (PCF) forms, both of which are amenable to modern molecular genetic approaches.

The secretory pathway in trypanosomes is much like other systems except that it is reduced in complexity, suggesting streamlining for efficient transport of major surface proteins, and also that it represents the basal requirements for secretory trafficking in eukaryotic cells (1, 2). Secretory proteins are imported into the endoplasmic reticulum (ER) where initial attachment of N-linked glycans occurs. There is a fairly typical eukaryotic ER quality control machinery to ensure proper protein folding (3–5), following which cargo is exported to the downstream Golgi compartment in COPII vesicles (6, 7). As protein cargo transits the Golgi compartment, N-glycans attached in the ER may be modified, and finally proteins are sorted to distinct post-Golgi destinations.

Likewise, N-glycosylation in trypanosomes is similar to that in other eukaryotes, but several aspects are sufficiently remarkable to warrant comment. The *T. brucei* genome encodes three oligosaccharyltransferases for attachment of N-glycans to consensus NXS/T sites (sequons) on nascent secretory proteins, TbSTT3A/B/C (8, 9). TbSTT3C is apparently not expressed in either BSF or PCF stages. TbSTT3B is constitutively expressed in both stages and selectively transfers triantennary oligomannose (Man₃GlcNAc₂) structures. TbSTT3A is upregulated in BSF trypanosomes and selectively transfers paucimannose structures missing the outer branches of the α1-6 mannose arm (biantennary Man₃GlcNAc₂) (see Fig. 7C). In addition to specificity for donor glycan structure, these oligosaccharyltransferases have distinct preferences for acceptor sites. TbSTT3A is selective for acidic glycosylation sites (sequon plus five flanking residues on either side) while TbSTT3B is nonselective for surrounding pI. Oligomannose glycans attached by TbSTT3B can be trimmed to triantennary Man₃GlcNAc₂ structures (see Fig. 7C), but because trypanosomes apparently lack Golgi mannosidase II, only the paucimannose structures transferred by TbSTT3A can be further processed to complex type oligosaccharides (10). Such processing can result in the addition of one or more N-acetyllactosamine (LacNAc or Galβ1-4GlcNAc) units to either arm of the trimannosyl core, and in extreme cases these can be processed to large heterogeneous poly-N-acetyllactosamine (pNAG) structures bearing an average of 54 LacNAc units per glycan (11). pNAG can be detected with tomato lectin (TL), which reportedly has a binding selectivity for three or more linear LacNAc units (12), and seems to be more prevalent on glycoproteins of the endosomal and lysosomal compartments (13). These findings have consequences for the stage specificity of N-glycosylation in trypanosomes. A given
glycoprotein bears only oligomannose N-glycans in PCF trypanosomes (14), but the same glycoprotein may bear oligomannose and/or complex type N-glycans in BSF trypanosomes (for experimental validation, see Fig. S1 in the supplemental material).

Protein disulfide isomerases (PDIs) are molecular chaperones involved in protein folding and quality control in the early secretory pathway of eukaryotic cells (15). They catalyze the formation of intra- and intermolecular disulfide bonds in newly synthesized secretory proteins. Critical to this function is the presence of one or two thioredoxin-like domains. The T. brucei genome encodes five putative PDI genes, one of which, TbPDI2 (Tb927.10.8230), initially known as BS2, is specifically upregulated in the BSF stage of the life cycle (5, 16). TbPDI2 contains two thioredoxin-like domains and is unusual in that it has 12 potential N-glycosylation sites. Most of these sites are utilized since treatment with peptide N-glycanase reduces the apparent molecular mass of the native protein (80 kDa) to a size consistent with that of the deduced polypeptide (55 kDa) (5). TbPDI2 is also unusual because it apparently localizes to the lysosome. Consistent with this finding is the localization and glycosylation status of TbPDI2 with the goal of yeast and mammalian orthologues without disrupting functionality (26, 27). Briefly, the 3′ end of the TbERD2 ORF (nt 163 to 657) was PCR amplified with an in-frame C-terminal fusion of the HA epitope (YPYD VPDYA) and flanking 5′ KpnI and 3′ EcoRI sites and inserted into the corresponding sites of pXS50neo (25) using 5′ HindIII and 3′ EcoRI sites. These reporter constructs were Xhol linearized for electroporation into pcDNA3.1 (TbERD2 RNAi). A construct was prepared for in situ chromosomal epitope tagging of TbERD2. The C terminus was targeted because tags have been placed in this position in both yeast and mammalian orthologues without disrupting functionality (19, 27). The TbERD2 3′ UTR (nt 213 to 744 relative to the stop codon) was PCR amplified with flanking 5′ Pal and 3′ SacI sites and inserted into the corresponding sites of the tagging vector. The entire construct was excised with KpnI/Sacl for electroporation into the BSF TsgT15Ty cell line (28). These cells have an in situ Ty-tagged glycophosphatransferase (TbGPT15) that serves as a specific marker for the Golgi complex. All cloning steps were confirmed by DNA sequencing. Electroporations were performed as described previously (7, 29), and clonal cell lines were derived by limiting dilution and selection with the appropriate antibiotic. Induction of TbERD2 double-stranded RNA (dsRNA) was achieved with 1 μg/ml of tetracycline.

**Materials and Methods**

**Maintenance of trypanosomes.** All Lister 427 strain bloodstream-form (M7t1.2 expressing VSG221) Trypanosoma brucei cell lines were grown in Ham’s medium with 10% fetal bovine serum (FBS) at 27°C (19). For experiments, all cells were harvested at mid-late log phase (BSF, 0.5 × 10^6 to 1 × 10^6 cells ml^−1; PCF, 0.5 × 10^6 to 1 × 10^6 cells ml^−1). **Antibodies and blotting reagents.** Rabbit anti-TbPDI2 was a generous gift of Derek Nolan (Trinity College, Dublin, Ireland). Rabbit anti-TbCatL, rabbit and mouse anti-BiP, and mouse monoclonal anti-p67 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-TbGPI8 was from the T. brucei genome. The BiPN:AVRG and BiPN:KQDL secretory reporter constructs were excised from pXS50neo (3) and inserted into pXS50neo (25) using 5′ HindIII and 3′ EcoRI sites. These reporter constructs were Xhol linearized for electroporation into pcDNA3.1 (TbERD2 RNAi). A construct was prepared for in situ chromosomal epitope tagging of TbERD2. The C terminus was targeted because tags have been placed in this position in both yeast and mammalian orthologues without disrupting functionality (26, 27). Briefly, the 3′ end of the TbERD2 ORF (nt 163 to 657) was PCR amplified with an in-frame C-terminal fusion of the HA epitope (YPYD VPDYA) and flanking 5′ KpnI and 3′ EcoRI sites and inserted into the corresponding sites of pXS50neo (25). Next, a portion of the TbERD2 3′ UTR (nt 213 to 744 relative to the stop codon) was PCR amplified with flanking 5′ Pal and 3′ SacI sites and inserted into the corresponding sites of the tagging vector. The entire construct was excised with KpnI/Sacl for electroporation into the BSF TsgT15Ty cell line (28). These cells have an in situ Ty-tagged glycophosphatransferase (TbGPT15) that serves as a specific marker for the Golgi complex. All cloning steps were confirmed by DNA sequencing. Electroporations were performed as described previously (7, 29), and clonal cell lines were derived by limiting dilution and selection with the appropriate antibiotic. Induction of TbERD2 double-stranded RNA (dsRNA) was achieved with 1 μg/ml of tetracycline.

**Quantitative real-time PCR.** Single-strand cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and an anchored oligo(dT) primer with 10 μg of total RNA harvested from log-phase parasite cultures using a Qiagen RNeasy Mini Kit (Valencia, CA). Real-time PCR was conducted using diluted cDNAs and Power SYBR green PCR Master Mix (Life Technologies, Carlsbad, CA) with oligonucleotide pairs spanning the trans-splice junction: spliced leader sense oligonucleotide (nt 5 to 25); TbGPI8 antisense oligonucleotide (5′ UTR nt −201 to −220 relative to the start codon; predicted splice site at −360 [tryppdb.org]); and TbERD2 antisense oligonucleotide (5′ UTR nt −137 to −154 relative to the start codon; predicted splice site at −260 [tryppdb.org]). Amplification was performed using an Applied Biosystems StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA). In each case postamplification melting curves indicated a single dominant product. The normalized TbERD2 gene expression levels were determined relative to the reference gene TbGPI8 and calculated by StepOne software, version 2.2.2. Reactions were performed in triplicate, and means ± standard errors of the means (SEM) for three biological replicates are presented.

**α-Mannosidase treatment of procyclic extracts.** Procyclic trypanosomes were radiolabeled (2 h) with [35S]Met-Cys (in the presence of FMK024 (20 μM); MP Biomedicals, Aurora, OH) as indicated in the text. Also as indicated (see Fig. S5 in the supplemental material) cells were pretreated (1 h) with tunicamycin (300 ng ml^−1; Sigma, St Louis, MO) and then radiolabeled in the continued presence of inhibitor. Subsequent immunoprecipitations of specific radiolabeled proteins from cell lysates were performed as described previously (22). Solubilization of primary immunoprecipitations and subsequent reconstituting for secondary pulldowns with TL-Bio, ECL-Bio, or ConA-Bio were performed as described by Kelley et al. (23). All lectin pulldowns were supplemented with 1 mM CaCl_2. Specific pulse and chase times are indicated in the text. Immunoprecipitates and pulldowns were fractionated by SDS-PAGE, and dried gels were analyzed by phosphorimaging using a Typhoon FLA 9000 with native ImageQuant software (GE Healthcare, Piscataway, NJ). Bands were quantified by volume analysis on regions of interest after subtracting background signal from equivalent unlabeled areas.

**TbERD2 RNAi, secretion, and epitope-tagging constructs.** A TbERD2 RNA interference (RNAi) construct was made by PCR amplifying the TbERD2 gene (Tb11.01.0410; entire open reading frame [ORF] plus 21 nucleotides [nt] of the 5′ untranslated region [UTR]) from Lister 427 genomic DNA. The amplicon was inserted into pZT7TI (24) using flanking 5′ BamHI and 3′ XhoI sites. The pZT7Te:ERD2 construct was NotI linearized for electroporation into cultured single-marker BSF trypanosomes. The BiPN:AVRG and BiPN:KQDL secretory reporter constructs were excised from pXS50neo (3) and inserted into pXS50neo (25) using 5′ HindIII and 3′ EcoRI sites. These reporter constructs were Xhol linearized for electroporation into pcDNA3.1 (TbERD2 RNAi). A construct was prepared for in situ chromosomal epitope tagging of TbERD2. The C terminus was targeted because tags have been placed in this position in both yeast and mammalian orthologues without disrupting functionality (26, 27). Briefly, the 3′ end of the TbERD2 ORF (nt 163 to 657) was PCR amplified with an in-frame C-terminal fusion of the HA epitope (YPYD VPDYA) and flanking 5′ KpnI and 3′ EcoRI sites and inserted into the corresponding sites of the tagging vector. The entire construct was excised with KpnI/Sacl for electroporation into the BSF TsgT15Ty cell line (28). These cells have an in situ Ty-tagged glycophosphatransferase (TbGPT15) that serves as a specific marker for the Golgi complex. All cloning steps were confirmed by DNA sequencing. Electroporations were performed as described previously (7, 29), and clonal cell lines were derived by limiting dilution and selection with the appropriate antibiotic. Induction of TbERD2 double-stranded RNA (dsRNA) was achieved with 1 μg/ml of tetracycline.
-specific mannosidase (ProZyme, Hayward CA) using manufacturer-supplied buffer (pH 5.0). The samples were then adjusted to final conditions for immunoprecipitation (radioimmunoprecipitation assay [RIPA] buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS) and subjected to sequential precipitation with anti-TbCatL and either TL:Bio or ConA:Bio.

**Enzymatic deglycosylation.** BSF trypanosomes were pulse radiolabeled with [35S]Met-Cys, and TbCatL (see Fig. S3 in the supplemental material), p67 (see Fig. S1), and TbPDI2 (see Fig. S1) polypeptides were specifically immunoprecipitated. Precipitates were solubilized in 1% SDS, collected by acetone precipitation, and then treated with either endoglycosidase H (EndoH) or peptide N-glycanase (PNGase) according to the manufacturer’s specifications (New England BioLabs, Ipswich MA). Samples were then fractionated by SDS-PAGE and visualized by phosphorimaging.

**Immunofluorescence microscopy.** All immunostaining was performed on formaldehyde-fixed, detergent permeabilized cells as previously described (30). Serial image stacks (0.2-μm Z-increment) were collected with capture times from 50 to 500 msec (100× PlanApo, oil immersion lens; 1.4 or 1.46 numerical aperture) on a motorized Zeiss Axioslager M2 equipped with a rear-mounted excitation filter wheel, a triple-pass (4’,6’-diamidino-2-phenylindole [DAPI]/fluorescein isothiocyanate [FITC]/Texas Red) emission cube, differential interference contrast (DIC) optics, and an Orca AG charge-coupled-device (CCD) camera (Hamamatsu, Bridgewater, NJ). All images were acquired with OpenLabs, version 5.0, software (Improvision Inc., Lexington, MA), and individual channel stacks were deconvolved by a constrained iterative algorithm, pseudocolored, and merged using Velocity, version 5.0, software (Improvision, Inc.). The x2y pixel precision of the red and green channels in this arrangement has been validated (see Fig. S1 in reference 7).

**RESULTS**

**TbPDI2 localizes to the ER.** The intracellular location of TbPDI2 in BSF trypanosomes was investigated using specific antibodies to endogenous BiP and p67 as markers for the ER and lysosome, respectively. As seen previously (25, 31), the ER presents as a continuous network throughout the cell, both anterior and posterior to the nucleus, while the lysosome presents as a single discrete vacuole in the region between the central nucleus and the posterior flagellar pocket, the latter indicated by the kinetoplast (Fig. 1A; see also Fig. S1A in the supplemental material). There is no overlap in the localization of these two well-characterized markers. An identical pattern of staining was obtained when TbPDI2 was imaged relative to p67 (Fig. 1B; see also Fig. S1B), and the overall distribution of TbPDI2 was highly suggestive of ER localization. Imaging of TbPDI2 relative to BiP (Fig. 1C; see also Fig. S1C) presented precisely overlapping patterns, confirming that the steady-state localization of endogenous TbPDI2 is in the ER, not the lysosome.

It may be that TbPDI2 does normally traffic to the lysosome but is degraded too rapidly to allow steady-state detection. Consequently, we investigated TbPDI2 biosynthesis and turnover by pulse-chase radiolabeling in the presence of FMK024, a selective inhibitor of lysosomal cysteine proteases (22, 32), and using two endogenous markers, p67 and TbCatL, as controls for lysosomal trafficking. p67 is a membrane glycoprotein that is synthesized as a 100-kDa ER glycoform (gp100). In BSF trypanosomes it is modified by pNAL addition in the Golgi complex (gp150) and is then cleaved upon arrival in the lysosome to quasi-stable fragments (gp75, gp42, and gp32) (25). TbCatL is a trypanosomal cathepsin L orthologue (previously known as trypanopain [33]) that is activated in the lysosome by removal of an N-terminal prodomain (21, 34). TbCatL has two N-glycosylation sites, and endoglycosidase H (Endo H) treatment indicates that both are occupied by oligomannose N-glycans (see Fig. S3 in the supplemental material). Both fragmentation of p67 and activation of TbCatL are blocked by FMK024. As expected, p67 in control cells was detected initially as gp100 and was substantially converted to gp42/gp32 at the end of the chase (Fig. 2, lanes 1 and 2). FMK024 had no effect on initial p67 synthesis but blocked subsequent turnover, leading to accumulation of the gp150 Golgi glycoform (Fig. 2, lanes 3 and 4). Also as expected, TbCatL in control cells was synthesized as a mixture of the precursor forms (I and X; the precise identity of X is uncertain) and the mature activated form (M) and was completely converted to the mature form at the end of the chase (Fig. 2, lanes 5 and 6). Note that much less mature TbCatL is seen with shorter pulse times (5 min) (see Fig. 5). In FMK024-treated cells the I and X precursors were detected initially, and X disappeared during the chase, but no activated TbCatL was seen (Fig. 2, lanes 7 and 8), consistent with inhibition of prodomain removal in the lysosome. TbPDI2 was synthesized as a sharp ~80-kDa species representing the core polypeptide of ~55 kDa plus up to 12 N-glycans predicted by the primary amino acid sequence (Fig. 2, lane 9). There was little apparent turnover of TbPDI2 during the chase period (Fig. 2, lane 10), and there was no change in size. Furthermore, despite its effect on p67 and TbCatL processing, FMK024 treatment did not result in higher recovery of TbPDI2 (Fig. 2, lanes 11 and 12) nor in its accumulation in the lysosome (immunofluorescence data not shown). Collectively, these localization

**FIG 1** Localization of TbPDI2. Cultured BSF trypanosomes were fixed/permeabilized and stained with combinations of primary antibodies: rabbit anti-BiP (green) and mouse anti-p67 (red) (A), rabbit anti-TbPDI2 (green) and mouse anti-p67 (red) (B); rabbit anti-TbPDI2 (green) and mouse anti-BiP (red) (C). Cells were stained with DAPI to detect nucleus and kinetoplast. In each case, three-channel summed stack projections of two representative cells (top and bottom) are presented. Cell outlines were traced from matched transmitted light images (data not shown). Nucleus (n), kinetoplast (k), endoplasmic reticulum (er), and lysosome (l) are indicated in panel A. Scale bar, 5 μm. Single-channel black and white images for each panel are presented in Fig. S2 in the supplemental material.
and turnover studies strongly indicate that TbPDI2 does not normally traffic to the lysosome in BSF trypanosomes.

**TbPDI2 localization is dependent on the TbERD2 retrieval receptor.** PDIs are molecular chaperones that assist in folding newly translocated secretory proteins; consequently, it makes sense that TbPDI2 is located in the ER. The mechanism for ER localization of soluble proteins, which has been documented in other systems (35), is a retrieval process whereby a C-terminal tetrapeptide signal (KDEL in mammals and HDEL in *Saccharomyces*) is recognized by a cognate membrane receptor, ERD2, in the cis-Golgi, followed by retrograde vesicular trafficking of the complex back to the ER. Querying the TriTryp genomic database (TriTrypDB [tritrypdb.org]) with the yeast ERD2 ORF (CAA84860) identified a single orthologue (*TbERD2*, Tb11.01.0410; BLASTP, 9.5e−36). Reciprocal querying of the nonredundant database with this gene strongly recognized multiple ERD2 orthologues. The trypanosome gene encodes a deduced polytopic membrane protein of 219 residues and ~26 kDa, consistent with ERD2 proteins in other systems. TbERD2 was HA tagged by *in situ* chromosomal recombinant, and its expression and location were determined relative to BiP and to a Ty-tagged Golgi glycosyltransferase, TbGT15Ty (28). Western blotting of the TbERD2HA/TbGT15Ty cell line revealed specific expression of a tagged protein of the expected size (Fig. 3A). In these cells TbERD2HA precisely colocalized with BiP throughout the ER and additionally presented as two prominent foci in the postnuclear region proximal to an area of the cell pe-

![FIG 2](image1.png) **FIG 2** Synthesis and turnover of TbPDI2. Cultured BSF trypanosomes were pulse (15 min)-radiolabeled with [35S]Met-Cys in the absence (−) or presence (+) of the lysosomal cysteine protease inhibitor FMK024 (FMK). At the indicated chase times, cell extracts were prepared, and p67, TbCatL, and TbPDI2 polypeptides were specifically immunoprecipitated as indicated. Immunoprecipitates were fractionated by SDS/PAGE (10^7 cell equivalents per lane), and radiolabeled polypeptides were visualized by phosphorimaging. All panels are from the same gel and phosphorimage and have been digitally separated for presentation. The p67 and TbCatL images were contrast-enhanced simultaneously; the TbPDI2 image was enhanced independently. The mobilities of p67 glycoforms (gp150-gp32), precursor (I and X), and mature (M) forms of TbCatL, and the mobility of TbPDI2 (P) are indicated on the left of the appropriate panels. Mobilities of molecular mass standards are indicated on the right (kDa). The small p67 species in FMK024-treated cells (lane 4, asterisk) has been seen previously (25), but its precise nature is unclear.

![FIG 3](image2.png) **FIG 3** Characterization of TbERD2 A. Total cell lysates from the TbGT15Ty (G) parental and TbGT15Ty/TbERD2HA (G/E) cell lines were analyzed by simultaneous Western blotting with rabbit anti-HSP70 and mouse anti-HA. The mobilities of endogenous HSP70 (H) and tagged TbERD2HA (E) and of molecular mass standards are indicated (kDa). (B) TbGT15Ty/TbERD2HA cells were fixed, permeabilized, stained, and imaged as described in the legend of **Fig. 1.** The following combinations of primary antibodies were used: for ERD2/ER, rabbit anti-HA (green) and mouse anti-BiP (red); for ERD2/Golgi complex, rabbit anti-HA (green) and mouse monoclonal anti-Ty (red). DAPI-stained nuclei (n) and kinetoplasts are indicated (left panels). Single-channel black and white images for each panel are presented in Fig. S4 in the supplemental material. (C) Specific TbERD2 message levels in control and silenced cells (24 h) were determined by quantitative reverse transcription-PCR as described in Materials and Methods (left). Normalized data are presented as means ± SEM (n = 3). The *TbERD2* RNAi cell line was cultured without (open circles) or with (closed circles) tetracycline to induce specific knockdown, and cell densities versus time were determined by hemocytometer (right). Cultures were adjusted daily to starting density. Data are presented as means ± SEM (n = 3). tet, tetracycline.
riphery called the flagellar attachment zone (Fig. 3B, left, arrowheads). This localization is suggestive of ER exit sites (ERES), of which there are typically two per interphase BSF cell (30). When imaged relative to TbGT15Ty, these foci were also seen to align closely with the Golgi compartment (Fig. 3B, right, arrowheads), which is always juxtaposed to matched ERES (30). These results indicate that while TbERD2HA is dispersed throughout the ER, it also has an elevated localization in discrete regions specialized for ER-Golgi compartment trafficking. RNAi silencing resulted in specific depletion of \( \text{TbERD2} \) mRNA (Fig. 3C, left) (87.7% ± 0.1%), with consequent delayed growth after 24 h (Fig. 3C, right), followed by complete cessation at 96 h (data not shown), indicating that \( \text{TbERD2} \) is essential for in vitro viability of BSF trypanosomes.

We previously used BiPN, the globular N-terminal domain of the endogenous ER molecular chaperone BiP, to demonstrate that the C-terminal tetrapeptide of TbPDI2 (KQDL) is sufficient to direct cellular retention (3). To confirm that TbERD2 mediates this process and that retention is specific to the ER, we constitutively expressed BiPN:KQDL in \( \text{TbERD2} \) RNAi cells. A matched cell line expressing BiPN:AVRG was used as a nonspecific secretory control. Silencing of \( \text{TbERD2} \) was induced, and the fates of the BiPN reporters were assessed by pulse-chase radiolabeling (Fig. 4). As expected, in control cells BiPN:AVRG is initially detected as an ~45-kDa cell-associated species that disappears over the chase period (Fig. 4A, top, lanes 1 to 5), concomitant with appearance in the external medium (Fig. 4A, top, lanes 6 to 10). Maximum final recovery of secreted BiPN:AVRG is ~50% (Fig. 4A, right), in close agreement with our prior work using the BiPN reporter in BSF trypanosomes (32). Full-length endogenous BiP (~72 kDa) remains cell associated throughout the chase period by virtue of its own C-terminal tetrapeptide (MDDL) (3). Also present in the initial cell fraction is newly synthesized VSG, the major endogenous glycosylphosphatidylinositol (GPI)-anchored surface protein that transiently associates with endogenous BiP in the ER (3). Silencing of \( \text{TbERD2} \) has no effect on the kinetics or extent of BiPN:AVRG export (Fig. 4A, bottom and right). In contrast, the BiPN:KQDL reporter is quantitatively retained in control cells (Fig. 4B, top, lanes 1 to 5), with little apparent secretion (Fig. 4B, top, lanes 6 to 10). Silencing of \( \text{TbERD2} \) dramatically reverses BiPN:KQDL retention (Fig. 4B, bottom, lanes 1 to 5), resulting in substantial reporter secretion (Fig. 4B, bottom, lanes 6 to 10). Quantification confirms the secretion of BiPN:KQDL when \( \text{TbERD2} \) is silenced, albeit not as rapidly as
BiPN:AVRG (Fig. 4B, right). The failure of endogenous BiP to be secreted when TbERD2 is silenced is expected since the C-terminal peptide binding domain of BiP can retard secretion when the native C-terminal tetrapeptide (MDDL) is deleted (3). Likewise no secretion of native TbPDI2 was detected (data not shown), presumably due to physical interaction with other nascent secretory proteins. Immunofluorescence microscopy indicates that the BiPN:KQDL reporter colocalizes precisely with TbpDI2, confirming targeting to the ER (Fig. 5). A similar but much less prominent pattern of intracellular staining was seen with BiPN:AVRG (data not shown), consistent with the constitutive secretion of this reporter. Collectively, these data confirm that the TbpDI2 C-terminal tetrapeptide KQDL is sufficient for ER retention in BSF trypanosomes and that this retention is mediated by the TbERD2 retrieval receptor. Furthermore, these results are incompatible with a lysosomal location for TbpDI2.

TbpDI2 is not modified with pNAL. TbpDI2 in BSF trypanosomes is thought to have pNAL-modified N-glycans because it binds tomato lectin (5). However, addition of pNAL to paucimannose N-glycans occurs as acceptor glycoproteins transit the Golgi compartment and often results in larger heterogeneous mature proteins, e.g., p67 gp150 (Fig. 2) (13, 22, 25). Thus, the ER localization of TbpDI2 and its sharp electrophoretic mobility are inconsistent with pNAL addition. To investigate this issue, BSF trypanosomes were pulse radiolabeled for 1 h, and sequential pulldowns were performed using specific antibodies for primary immunoprecipitation and, following solubilization of precipitates under denaturing conditions, TL for secondary pulldown of all p67 glycoforms, confirming that TL reactivity was directed at N-linked glycans (see Fig. S5 in the supplemental material). In contrast and as expected, the typical spectrum of precursor and mature TbcatL species (bearing only oligomannose glycans) were seen in primary immunoprecipitates (Fig. 6A, lane 4) but not in secondary pulldowns with TL (Fig. 6A, lane 5). Finally, TbpDI2 was detected as a single sharp species in primary immunoprecipitates and was efficiently recovered by secondary pulldown with TL (Fig. 6A, lanes 7 and 8). As with p67, TL reactivity of TbpDI2 was inhibited by chitin hydrolysatasy (Fig. 6A, lane 9) and biotin (data not shown). These data confirm the prior finding of Rubotham et al. (5) that TbpDI2 is reactive with tomato lectin, and they are apparently consistent with pNAL modification.

However, the reactivity of TL with the gp100 p67 glycoform is troubling since this species represents newly synthesized protein in the ER, which should not be pNAL modified. Therefore, to investigate how quickly p67 and TbpDI2 TL reactivity are acquired, these experiments were repeated with a 5-min pulse-label to ensure that radiolabel was incorporated only into newly synthesized ER polypeptides (Fig. 6B). As expected, p67 was detected mostly as the gp100 glycoform in primary immunoprecipitates (Fig. 6B, lane 1), which was again substantially and unexpectedly recovered in the secondary TL pulldown (Fig. 6B, lane 2). Likewise, TbpDI2 from the primary immunoprecipitate (Fig. 6B, lane 7) was efficiently recovered in the subsequent TL pulldown (Fig. 6B, lane 8). No TL reactivity was seen with newly synthesized TbcatL species (Fig. 6B, lane 5).

These surprising results seemingly suggest that both newly synthesized p67 and TbpDI2 acquire TL-reactive pNAL-containing N-glycans very rapidly without accessing the downstream N-gly-
can processing machinery in the Golgi complex. To challenge this interpretation, we repeated the sequential pulldowns using *Erythrina cristagalli* lectin (ECL) as the secondary reagent. ECL has a binding preference for single LacNAc units (37) and should react with any pNAL-containing glycoproteins. Following a 1-h pulse-label, ECL detected the mature gp150 p67 glycoform and derivative gp75 and gp42 cleavage products, and this reactivity was inhibited with excess lactose (Fig. 6C, lanes 2 and 3), confirming the presence of LacNAc in the N-glycans of these polypeptides. As expected, ECL did not react with either p67 gp32 or TbCatL (Fig. 6C, lanes 2 and 5). Most importantly, ECL also failed to detect p67 gp100 or TbPDI2 in secondary pulldowns (Fig. 6C, lanes 5 and 8), confirming that these glycoproteins do not have LacNAc-containing N-glycans. Following a 5-min pulse-label, no secondary ECL reactivity was seen for any species of p67, TbCatL, or TbPDI2 (Fig. 6D, lanes 2, 5, and 8). The reactivity of mature p67 glycoforms with both TL and ECL confirms the bona fide addition of pNAL units as this protein transits the Golgi compartment. Conversely, the failure of "mature" TbPDI2 to react with ECL indicates that subsequent LacNAc or pNAL modification of paucimannose N-glycans attached in the ER does not occur. Most importantly, these results indicate that neither newly synthesized p67 nor TbPDI2 has exposed LacNAc moieties and, consequently, that the observed reactivity of these proteins with TL cannot be due to the rapid acquisition of larger pNAL structures.

**Tomato lectin binds abbreviated N-glycans.** One explanation for the surprising reactivity of TL with newly synthesized ER glycoproteins is that it may bind abbreviated N-glycan structures. TL has been shown to bind to the residual Man₅GlcNAc₂ [Man₁₋₃(Man₁₋₆)Manβ₁₋₄GlcNAcβ₁₋₄GlcNAc] pentasaccharide of complex N-glycans treated with sialidase, β-galactosidase, and β-hexosaminidase and to the exposed ManGlcNAc₂ (Manβ₁₋₄GlcNAc) trisaccharide core of oligomannose N-glycans treated with α-mannosidase (38). This raises the possibility that TL binds to native Man₅GlcNAc₂ paucimannose oligosaccharides [Man3-1(Man1-3)Manβ1-4GlcNAcβ1-4GlcNAc] attached to acidic glycosylation sites of newly synthesized p67 and TbPDI2 in BSF trypanosomes (Fig. 7C). To assess this possibility, we tested the binding of TL to oligomannose N-glycans on TbCatL from PCF trypanosomes following treatment with β-mannosidases. TbCatL has two EndoH-sensitive oligomannose N-glycans (see Fig. S3 in the supplemental material), and furthermore, primarily trimmed Man₅GlcNAc₂ oligomannose structures [Manα₁₋₆(Manα₁₋₆)Manα₁₋₆(Manα₁-
labeled N-glycan cores (A) Procytic trypansomes were pulse-radiolabeled (2 h) with [35S]Met-Cys in the presence of FMK024 to inhibit maturation of TbCatL. Cell extracts were prepared and treated overnight without (control) or with jack bean α-mannosidase (JBAM). Lysates were then used for primary immunoprecipitation with anti-TbCatL (α) and subsequent secondary pulldown with TL (T) or ConA (C) without (−) or with (+) competing chitin hydrolysate (1/1,000) or α-methylmannoside (200 mM), respectively. Equivalent amounts of all samples were fractionated by SDS-PAGE (107 cell equivalents per lane), and radiolabeled polypeptides were visualized by phosphorimaging. The mobilities of immature TbCatL proprotein (I) and molecular mass markers are indicated on the left (kDa). A single representative phosphorimage is presented. (B) The experiment is identical to that described for panel A except that Aspergillus saitoi α1–2-specific mannosidase (ASAM) was used. (C) Diagrams of relevant N-linked oligosaccharide structures (left to right): initial Man3GlcNAc2, and subsequently trimmed Man3GlcNAc2 oligomannose structures found in both PCF and BSF trypansomes; the Man3GlcNAc2 product of jack bean α-mannosidase digestion; and the biosynthetic Man6GlcNAc2, paucimannose structure found in BSF trypanosomes. Black squares, N-acetylglucosamine; gray circle, β-linked mannose; white circles, α-linked mannose. Specific α1–2 (2), α1–3 (3), and α1–6 (6) linkages are indicated in the left panel. Not shown are the transient terminal glucose residues found on the α1-3 branch of recently attached full and partially trimmed oligomannose structures and paucimannose structures in T. brucei (42).

3)Manβ1–4GlcNAcβ1–4GlcNAc] (Fig. 7C) are found on all glycoproteins in PCF trypanosomes (14, 39). A single polypeptide corresponding to the full-length doubly N-glycosylated TbCatL proprotein was synthesized in FMK024-treated PCF trypansomes (Fig. 7A, lane 1). This species was specifically detected in secondary pulldowns with ConA but not with TL (Fig. 7A, lanes 2 versus 4). Treatment with jack bean α-mannosidase slightly reduced the size of the TbCatL proprotein (Fig. 7A, lane 6), consistent with removal of the α1–3 and α1–6 mannose arms of both N-glycans (Fig. 7C). The TbCatL protein was then specifically recognized in secondary pulldown with TL (Fig. 7A, lanes 7 and 8), and concomitantly detection with ConA was reduced (Fig. 7A, lanes 9 and 10). Similar treatment with Aspergillus saitoi α1–2-specific mannosidase did not enhance reactivity with TbCatL, emphasizing that fully trimmed triantennary Man3GlcNAc2 is not a TL ligand (Fig. 7B). These results confirm that TL can bind to abbreviated core structures derived from oligomannose N-glycans (Fig. 7C). Both p67 (see Fig. S1 in the supplemental material) and TbPDI2 (5) from BSF trypanosomes contain mixtures of oligomannose (Endo H-sensitive) and paucimannose (Endo H-resistant) N-glycans, and these results suggest that TL may also bind the later structures.

**DISCUSSION**

TbPDI2 is a trypanosomal protein disulfide isomerase orthologue that is specifically upregulated in pathogenic bloodstream-form trypanosomes (5, 16). It has been localized to the lysosome, and like other proteins of the lysosome/endosomal system in BSF trypansomes, has been thought to contain pNAL-modified N-glycans based on reactivity with TL. These observations suggested that TbPDI2 plays a role in bloodstream-form pathogenesis, perhaps by aiding in the unfolding and degradation of host-derived trypanolytic antibodies that are rapidly internalized from the cell surface and delivered to the lysosome (5). However, two additional considerations are inconsistent with these conclusions. First, TbPDI2 has a C-terminal tetrapeptide signal (KQDL) that is sufficient to mediate cellular retention of a recombinant soluble secretory reporter in PCF trypanosomes (3). This suggests that TbPDI2 is retained in the ER by a classic retrieval mechanism although this had not been formally proven at the outset of this investigation. Second, while the reactivity of TbPDI2 with TL is well documented (5), the apparent size of the protein is quite uniform, as judged by electrophoretic mobility, suggesting a remarkably homogeneous pattern of glycosylation for a protein containing up to 12 N-glycans. In contrast, pNAL modifications are usually quite heterogeneous, containing on average ~54 LacNAc units per glycan (11), which can result in very diffuse apparent sizes, as seen with the p67 gp150 glycoform. In light of these seemingly contradictory lines of evidence, we have reevaluated the localization and glycosylation status of TbPDI2.

Our results indicate that TbPDI2 actually resides in the ER, where it colocalizes overwhelmingly with the definitive ER marker BiP. No evidence of lysosomal localization was seen even when possible turnover was blocked with a potent lysosomal thiol protease inhibitor. Furthermore, the BiPN:KQDL reporter localized to the ER, consistent with our prior results and demonstrating that the PDI C-terminal tetrapeptide is a bona fide ER localization signal. To confirm that ER localization is mediated by the classic eukaryotic retrieval mechanism, we have identified and characterized TbERD2, the trypanosomal orthologue of the common eukaryotic retrieval receptor. Consistent with this function, TbERD2 is found throughout the ER in BSF trypanosomes but most prominently in close association with ER exit sites and the Golgi compartment. Critical reduction in TbERD2 expression results in secretion of the BiPN:KQDL reporter, confirming that localization of endogenous soluble ER proteins such as TbPDI2 and BiP in trypanosomes is signal specific and TbERD2 mediated.

As did Rubotham et al. (5), we find that TbPDI2 is reactive with TL, implying the presence of pNAL. This glycan modification occurs as glycoproteins bearing paucimannose N-glycans (Fig. 7C) are exposed to specific glycosidases and glycosyltransferases while transiting the Golgi compartment. This is theoretically possible
since paucimannose oligosaccharides are attached to acidic glyco-sylation sites (9), and most of the TbPDI2 sequons have pI < 6.0 (see Table S1 in the supplemental material). Furthermore, TbPDI2 in BSF trypanosomes has EndoH-resistant N-glycans, consistent with paucimannose structures (5). However, TbPDI2 would first have to reach the Golgi compartment by vesicular transport, and both its ER localization and the rapid onset of TL reactivity argue against this likelihood. We did consider the possibility that the ER retrieval process would allow sufficient trans-ient exposure of TbPDI2 to Golgi complex-processing enzymes for minimal pNAL modification to occur while still maintaining both a steady-state ER localization and sharp electrophoretic mo-bility. However, expression of a dominant negative Tbtūr1 mu-tant, which blocks vesicular trafficking from the ER (7), had no effect on the early onset of TL reactivity (data not shown). Fur-thermore, TL reactivity was also seen with the p67 gp100 ER gly-cofor, indicating that this phenomenon is not restricted to TbPDI2 and that retrieval is not required. Finally, newly synthe-sized TbPDI2 and gp100 were not recognized by ECL, arguing strongly against the existence of the terminal LacNac units that should be present if these proteins were modified with pNAL. Nevertheless, competitive inhibition with chitin hydrolysate and the failure to recognize undeglycosylated polypeptides synthesized in tunicamycin-treated cells together indicate that TL binds to some kind of N-glycan structures.

Given these conflicting sets of data we explored the alternative explanation that TL may recognize non-pNAL glycotopes on paucimannose N-glycans of both TbPDI2 and p67 gp100. This possibility was suggested by the facts that TL does not recognize the trimmed triantennary Man,GlcpNAc2 oligomannose struc-tures found on TbCatL and that TL has been previously shown to recognize the Man,GlcpNAc2 core of abbreviated N-glycans gener-ated by exoglycosidase treatment (38). Consistent with this possi-bility, jack bean α-mannosidase treatment converted the oligomannose N-glycans of TbCatL to TL-reactive structures. These are most likely the residual Man,GlcpNAc2 core, but this cannot be definitively derived from the data presented here. We propose that this minimal epitope within paucimannose N-glycans is recog-nized by TL but is sterically blocked by the presence of either or both of the outer branches of the α1-6 arm in triantennary oligomannose N-glycans (Fig. 7C). In addition, our pulldown protocol involves a denaturation step that likely facilitates the secondary lectin-ligand interaction by exposing cryptic glycotopes in folded proteins. Nevertheless, native TbPDI2 clearly binds to TL (5), presumably because one or more paucimannose N-glycans are suffi-ciently exposed to allow physical interaction.

Our findings may also resolve contradictory evidence on the reported TL reactivity of native transferrin receptor (TfR) in BSF trypanosomes. TfR was one of the glycoproteins originally shown to be TL reactive by Nolan et al. (13), suggesting that it contained pNAL-modified N-glycans. However, more recently TfR was shown by sensitive analytical methods to contain paucimannose oligosaccharides without pNAL modification (40). Our model of TL binding to paucimannose N-glycans may account for these disparate observations. Consistent with this, we have found using our sequential precipitation protocol with anti-TfR antibody and TL that both the ESAG6 and ESAG7 subunits of TfR are TL reac-tive (data not shown).

In summary, our results serve as a cautionary note about the utility of TL in assessing addition of pNAL to N-glycans in BSF trypanosomes. TL binding alone is clearly insufficient to make definitive judgments, and additional criteria must be applied, in-cluding assessment of electrophoretic mobility and reactivity with other lectins such as ECL and ricin. This does not mean that TL is without utility. Indeed, in our hands it is superior to both wheat germ agglutinin and ConA as a quantitative marker of endocytic delivery to the lysosome (41) (unpublished observations). In addition, with careful application, TL may be useful in demonstrating the presence of paucimannose N-glycans on glycoproteins in BSF trypanosomes. Finally, we believe that these findings resolve the contradictory observations regarding the location and glyco-sylation of TbPDI2. TbPDI2 is part of the secretory quality control machinery and is localized to the ER by a classic eukaryotic C-ter-minal tetrapeptide-specific and TbERD2-mediated retrieval pro cess. To our knowledge, trypanosomes are currently the most an- cient eukaryotic lineage in which this machinery has been formally demonstrated. The fact that TbPDI2 is upregulated in BSF try-panosomes (5, 16) suggests a role in the synthesis and folding of stage-specific secretory proteins—most likely the abundant vari-ant surface glycoproteins—the most critical virulence factor influ-encing the success of this important human pathogen.

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