The unfolded protein response (UPR) is a stress mechanism to cope with misfolded proteins in the early secretory pathway, the hallmark being transcriptional upregulation of endoplasmic reticulum (ER) molecular chaperones such as BiP and protein disulfide isomerase. Despite the lack of transcriptional regulation and the absence of the classical UPR machinery, African trypanosomes apparently respond to persistent ER stress by a UPR-like response, including upregulation of BiP, and a related spliced leader silencing (SLS) response whereby SL RNA transcription is shut down. Initially observed by knockdown of the secretory protein translocation machinery, both responses are also induced by chemical agents known to elicit UPR in mammalian cells (H. Goldshmidt, D. Matas, A. Kabi, A. Carmi, R. Hope, S. Michaeli, PLoS Pathog 6:e1000731, 2010, http://dx.doi.org/10.1371/journal.ppat.1000731). As these findings were generated primarily in procyclic-stage trypanosomes, we have investigated both responses in pathogenic bloodstream-stage parasites. RNA interference (RNAi) silencing of the core translocon subunit Trypanosoma brucei Sec61α (TbSec61α) failed to induce either response. Interestingly, cell growth halted within 16 h of silencing, but sufficient TbSec61α remained to allow full competence for translocation of nascent secretory proteins for up to 24 h, indicating that replication is finely coupled with the capacity to synthesize and transport secretory cargo. Tunicamycin and thapsigargin at concentrations compatible with short-term (4 h) and long-term (24 h) viability also failed to induce any of the indicators of UPR-like or SLS responses. Dithiothreitol (DTT) was lethal at all concentrations tested. These results indicate that UPR-like and SLS responses to persistent ER stress do not occur in bloodstream-stage trypanosomes.

While the basic SLS response is well documented, it is less clear how knockdowns that should actually prevent the import of nascent secretory proteins, from a putative ER-associated kinase (TbeIFK3) to the SL transcription preinitiation complex has been described (11). Most of this genetic and molecular work was performed in procyclic culture forms (PCF), corresponding to insect midgut parasites, but loss of SL RNA was also observed in mammalian bloodstream-form (BSF) cells upon TbSec63 knockdown (4). Knockdown of TbSRα and TbSec61α was not attempted in BSF cells.

While the basic SLS response is well documented, it is less clear how knockdowns that should actually prevent the import of nascent secretory proteins, and thereby reduce total secretory flux,
constitute persistent ER stress. However, pharmacological treatment that induces bona fide UPR in other eukaryotic systems apparently also induces SLS responses in both PCF and BSF parasites. In each case, short-term acute treatment with DTT (4 mM, 4 h) increased steady-state levels of BiP and reduced steady-state levels of SL RNA (4). DTT also elevated expression of tSNAP42 and induced its relocation within the nucleus in PCF trypanosomes (tSNAP42 phenotypes in BSF cells were not reported). These responses to DTT treatment are the only direct experimental link between a classic UPR and the SLS response. However, this finding has been challenged by other investigators (12). Koumandou et al. found that 4 mM DTT is highly toxic to BSF trypanosomes but were able to assess the effect of a lower concentration (1 mM, 4 h) (12). They found no change in BiP expression at either the transcript or protein level. Also tested was tunicamycin, an inhibitor of N-glycan synthesis (13), at levels that did not impact short-term viability (5 μg/ml, 24 h). Note that in our hands this concentration, which is 25-fold higher than that required to block N-glycosylation in BSF trypanosomes, is 100% lethal at 24 h. Again, no effect on BiP expression was detected. This study did not look for induction of an SLS response, nor did it look at PCF trypanosomes. In addition to these published data, we can add anecdotal results. When we originally developed the anti-BiP antibody used by all of these investigators (14), we attempted to modulate BiP expression by DTT treatment. These experiments were all negative due to acute toxicity for cultured trypanosomes (J. D. Bangs, unpublished results).

In this report we investigate the existence of a UPR-like response, i.e., upregulation of ER molecular chaperone expression, in BSF trypanosomes in the context of ablation of the core translocon subunit TbSec61α, as well as both short-term acute and long-term subacute pharmacological treatments. In the process, we attempt to resolve the contradictory results obtained with DTT treatment. We also assess the ability of these treatments to generate an SLS response, i.e., upregulation of tSNAP42 and downregulation of SL RNA. Our overall results call into question the status in BSF trypanosomes of UPR-like and SLS responses as phenom-ena that can be initiated with persistent ER stress.

MATERIALS AND METHODS

Cell lines and culture. All work was carried out with the bloodstream-form Lister 427 strain of *Trypanosoma brucei brucei* (MITat1.2 expressing VSG221) grown in HMI-9 medium (15). A derivative tetracycline-responsive single-marker cell line (16) was used for all RNAi experiments. All phenotypic assays were performed with freshly harvested cells from mid- to late-log-phase cultures (0.5 × 10⁶ to 1.0 × 10⁶ per ml). Pharmacological treatments were all initiated by addition of reagent (DTT, thapsigargin, or tunicamycin) from 1,000× stocks in dimethyl sulfoxide (DMSO) to final concentrations indicated in the text. Viable cell densities were determined by visual counting with a hemocytometer. Viability was rigorously scored only if motility was apparent, regardless of actual morphological condition.

**Immunological reagents.** Rabbit anti-VSG221, anti-BiP, and anti-Hsp70 have all been described elsewhere (17, 18). Rabbit anti-TbPD12 was a generous gift of Derek Nolan (Trinity College, Dublin, Ireland). Loading conditions for Western blotting, i.e., culture volume versus cell numbers, are indicated in the relevant figure legends. Secondary reagents were IRDye680- and IRDye800-conjugated goat anti-rabbit IgG (Li-Cor, Lincoln, NE). Secondary reagents for immunofluorescent imaging were species-specific Alexa-conjugated goat anti-IgG as appropriate (Molecular Probes, Eugene, OR).

**Immunoprecipitation of radiolabeled polypeptides.** Cells were radiolabeled with [35S]methionine-cysteine, detergent extracted, immunoprecipitated with specific antibodies, fractionated by SDS-PAGE, and analyzed by phosphorimaging as described previously (17, 18).

**Immunoblotting.** Gels were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Trans-Blot Turbo apparatus (Bio-Rad, Hercules, CA). Membranes were blocked and probed with appropriate dilutions of primary and secondary antibodies in Odyssey blocking buffer (Li-Cor). All washes were with phosphate-buffered saline (PBS)–0.5% Tween 20. Quantitative fluorescent signals were scanned on an Odyssey CLx Imager (Li-Cor).

**Data analyses.** Fluorescent blot scans were quantified with ImageJ software (http://imagej.nih.gov/ij/). For analysis of specific band intensities, signals were corrected by subtraction of the signal from equivalent unlabeled areas of each lane. All subsequent data management was performed with Prism, version 4, software (GraphPad Software, Inc., San Diego, CA).

**qRT-PCR.** Transcript levels were determined using quantitative reverse transcription-PCR (qRT-PCR). Total RNA was isolated from log-phase cultures using an RNeasy minikit (Qiagen, Valencia, CA). RNA was treated with DNase 1 on column using an RNase-Free DNase Set (Qiagen), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad) with combined oligo(dT) and random hexamer priming. qRT-PCR was performed using diluted cDNAs and Power SYBR green PCR master mix (Life Technologies, Carlsbad, CA) with oligonucleotide pairs targeting the following transcripts: spliced leader RNA (nt 26 to 108), 7SL RNA (GeneDB [www.genedb.org] accession number Tb927.8.2861, nt 147 to 221), TbSNAP42 (Tb927.5.3910, nt 703 to 811), TbSec61α (Tb927.11.6230, nt 222 to 326), BiP (Tb927.2.7200, nt 940 to 1080), TbZFP3 (Tb927.3.720, nt 241 to 301). Amplification was performed using an Applied Biosystems StepOne real-time PCR system (Life Technologies, Carlsbad, CA). For each transcript, postamplification melting curves indicated a single dominant product. Spliced leader RNA transcripts were normalized to 7SL RNA, while all other protein coding gene transcript levels were normalized to the TbZFP3 level. All calculations and normalizations were done using StepOne software, version 2.2.2. All reactions were performed in technical triplicate, and means ± standard errors of the means (SEM) for three biological replicates are presented.

**TbSec61α RNAi and epitope tagging.** A TbSec61α stem-loop RNAi construct was generated using the pLEW1005X:Pex11 stem-loop vector (19). A 581-bp fragment (bp 712 to 1292) of the TbSec61α open reading frame (ORF) was PCR amplified with nested 5’ Xhol/BamHI and 3’ Ascl/Ndel sites. The PCR product was sequentially inserted postdigestion upstream of the Pex11 stuffer using BamHI/Ascl and downstream in the opposite orientation using Xhol/Ndel. All cloning steps were confirmed by sequencing, and the resultant plasmid was digested with NotI for transfection of the single-marker BSF cell line by electroporation as described previously (20, 21). Clonal cell lines were derived by limiting dilution and selection with phleomycin. Induction of TbSec61α double-stranded RNA was achieved by addition of 1 μg/ml of tetracycline.

An in situ TbSec61α N-terminal Ty tagging construct was prepared using pNNT4pm, a derivative of pX66pm (19) (details available on request). The TbSec61α 5’ untranslated region (UTR; nt −1 to −490 relative to the TbSec61α start codon) was PCR amplified with flanking 5’ Clal and 3’ Ascl sites and inserted upstream of the puromycin resistance (Pur+) cassette in pNNT4pm. Next, the TbSec61α ORF was PCR amplified (nt 3 to 1446) with an N-terminal in-frame fusion of a Ty epitope tag (MEVHTN QDPLD; start codon added) with flanking 5’ SnaBl and 3’ MfeI sites and inserted into the corresponding sites of the pNNT4pm TbSec61α 5’ UTR construct above. The completed tagging construct consisting of (5’ to 3’) the TbSec61α 5’ UTR, the Pur+ cassette, the β-α-tubulin intergenic region, and the N-terminal Ty-tagged TbSec61α ORF was excised with Clal/
MfeI for transfection into a TbSec61α RNAi cell line with puromycin selection as described above.

**Epifluorescence microscopy.** Immunofluorescence (IF) microscopy was performed with formaldehyde-fixed/detergent-permeabilized cells as described by Tazeh and Bangs (22). Cells were also stained with 4,6-diamidino-2-phenylindole (DAPI; 0.5 μg ml⁻¹) to reveal nuclei and kinetoasts. Serial image stacks (0.2-μm z-increment) were collected with capture times from 100 to 500 msec (100× Plan Apo oil immersion lens, 1.46 numerical aperture) on a motorized Zeiss Axiosimager M2 stand equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/fluorescein isothiocyanate [FITC]/Texas Red) emission cube, differential interference contrast (DIC) optics, and an Orca ER charge-coupled-device (CCD) camera (Hamamatsu, Bridgewater, NJ). Images were collected with Volocity, version 6.1. Acquisition Module (Imagination Inc., Lexington, MA), and individual channel stacks were deconvolved by a constrained iterative algorithm, pseudocolored, and merged using the Volocity, version 6.1, Restoration Module. Unless otherwise stated, all images presented are summed stack projections of merged channels. The xy2 pixel precision of this arrangement has been validated previously (see reference 21 and Fig. S1 therein).

**RESULTS**

**TbSec61α silencing does not induce a UPR-like or SLS response.** We first asked if either UPR-like or SLS responses could be induced in BSF trypanosomes by conditional RNAi silencing of the machinery for ER import of secretory cargo. For this work we focused on TbSec61α as it is the core subunit of the translocon responsible for import of all secretory proteins (23). TbSec61α silencing halted cell growth within 16 h of induction (Fig. 1A), and knockdown of TbSec61α message was ~90% at 24 h (Fig. 1C). However, viability and cell morphology (see Fig. 3) remained unaffected at 24 h, allowing a window of ~8 h during which metabolically intact cells could respond. Analysis of DAPI-stained cells revealed that there was no difference in the distributions of kinetoplast and nuclear copy numbers, indicating that growth arrest occurs at all stages of the cell cycle (Fig. 1B). Loss of TbSec61α had no effect on the level of BiP message, but, strikingly, TbPDI2 message was reduced ~70% (Fig. 1C). Consistent with these results, steady-state BiP protein was unaltered, but TbPDI2 protein was noticeably reduced (Fig. 1D). We cannot explain the negative impact on TbPDI2 expression, which was observed consistently, but nevertheless these results indicate that silencing the translocation machinery does not induce a UPR-like upregulation of ER chaperones in BSF trypanosomes. Also unaffected was mRNA for the major secretory proteins: BiP, VSG, p67, and TbCatL. VSG is the major secretory protein of BSF trypanosomes, comprising ~10% of total cellular protein (24). It is glycosolphosphatidylinositol (GPI) anchored and in the strain used in this work has two N-linked glycans (25). p67 is a lysosomal type I membrane glycoprotein (26). It is synthesized as a 100-kDa ER precursor with 14 N-glycans (gp100) and is processed in the Golgi compartment to a gp150 species, which is subsequently cleaved to quasi-stable gp42 and gp32 species in the lysosome. TbCatL is a luminal lysosomal cathepsin L orthologue. It is synthesized as a precursor doublet (1, 53 kDa; X, 50 kDa), each with two N-glycans, that is converted upon arrival in the lysosome to a mature enzyme (44 kDa) by removal of a prodomain (17, 19). Failure to translocate any of these secretory proteins should be evidenced by obvious and interpretable changes in electrophoretic mobility due to failure in signal sequence cleavage, addition of N-glycans and GPI anchors, and/or

![FIG 1 RNAi Silencing of TbSec61α](http://ec.asm.org/)

**A. Growth**. The TbSec61α RNAi cell line was cultured without (-) or with (+) tetracycline (tet) to induce specific knockdown. (A) Cell density over time was determined by hemocytometer, and cells were adjusted to starting density daily. Data are presented as means ± SEM (n = 3). Cell growth terminated at ~16 h, and all subsequent phenotypic analyses were performed at 24 h when morphology and motility were still normal. (B) Kinetoplasts (K) and nuclei (N) per cell were quantified with DAPI-stained cells. Data are means ± SEM for two biological replicates (200 cells each). (C) cDNA was prepared, and qRT-PCR was performed to determine relative levels of specific mRNA and SL RNA as indicated. Signals for protein-coding mRNAs were normalized to TbzP133 signal, and the signal for SL RNA was normalized to that of 7SL RNA. All signals are ratios calculated relative to the equivalent normalized signal from nonsilenced cells. Means ± SEM are presented for three biological replicates. (D) Whole-cell extracts (10^6 cell equivalents/lane) were transferred to membranes and subjected to simultaneous probing with anti-VSG and anti-BiP (V and B) or with anti-TbPDI2 (P) or anti-Hsp70 (H) antibodies as indicated. Representative blots from one of three independent biological replicates are presented. Mobilities of molecular mass standards are indicated. (E) BIP signals for control and silenced cells were quantified and normalized to the corresponding Hsp70 signals (B/H). Means ± SEM are presented for four biological replicates.
FIG 2 Effect of TbSec61α silencing on translocation. (A) TbSec61α RNAi cells were cultured with (+) or without (−) tetracycline for 24 h. Cells were then washed and metabolically radiolabeled for 1 h with [35S]Met-Cys in the presence of tetracycline. As indicated, endogenous proteins were immunoprecipitated with specific antibodies, fractionated by SDS-PAGE, and subjected to phosphorimaging. Mobilities of specific polypeptides are indicated as described in the text and in the legend of Fig. 1. Precursors (I, X) and mature (M) TbCatL polypeptides are also indicated. Note that a portion of total VSG representing the most recently synthesized polypeptide coprecipitates with BiP as a consequence of its function as an ER molecular chaperone (37). All lanes are from the same scan of a single gel and were digitally processed simultaneously. The individual panels were then excised to allow convenient labeling. For VSG, 10⁵ cell equivalents/lane were used; for all others, 5 × 10⁶ cell equivalents/lane were used. (B) Cell extracts (10⁵ cell equivalents/lane) from the parental RNAi cell line (P−) and the derivative Ty-tagged (P+) cell line were immunoblotted with anti-Ty or anti-Hsp70 as indicated. Mobilities of Ty-TbSec61α (Ty), Hsp70 (H), and molecular mass standards are indicated. (C) TbSec61α silencing was induced in the Ty-TbSec61α cell line. Aliquots were harvested at the indicated time points and analyzed as described for panel B.

post-ER glycan processing events. Cytoplasmic Hsp70 was used as a control for global translation. TbSec61α-silenced cells were pulse-radiolabeled (1 h), cell extracts were prepared, and the endogenous reporter polypeptides were specifically immunoprecipitated and analyzed by SDS-PAGE and phosphorimaging (Fig. 2A). Surprisingly, there was no effect on the synthesis and posttranslational processing of any of these reporters. Furthermore, the levels of translation for each were unaffected, consistent with the lack of impact of TbSec61α silencing on the mRNA levels for each protein.

These results indicate that even after 8 h of stalled growth due to TbSec61α silencing, cells retain sufficient capacity to translocate a full and normal complement of endogenous secretory proteins. To test this, TbSec61α was Ty epitope tagged by in situ chromosomal recombination in the TbSec61α RNAi cell line. Western blots indicate specific expression of the tagged protein (Fig. 2B). Note that Ty-TbSec61α mobility is aberrantly small (expected molecular mass, ~54 kDa), as often occurs with multipass integral membrane proteins. Loss of tagged protein was followed during specific RNA silencing (Fig. 2C). By 16 h, when cell growth had stopped, TbSec61α protein levels were reduced dramatically, and thereafter up to 24 h they remained relatively constant. Immunofluorescent imaging confirmed that TbSec61α colocalizes precisely with BiP in the ER (Fig. 3). No staining was observed in the parental RNAi cell line (data not shown), indicating that ER localization is specific for tagged TbSec61α despite the considerable nonspecific reactivity seen in immunoblots (Fig. 2B, P−). These combined results indicate that functional translocons are widely distributed throughout the ER and confirm that sufficient TbSec61α protein remains to constitute functional translocons well after cell growth ceases.

Pharmacological treatments do not induce a UPR-like response. We next assessed the trypanocidal and/or trypanostatic properties of pharmacological reagents typically used to induce UPR in mammalian cells (Fig. 4A). In our hands both of the DTT concentrations used by prior investigators, 4 mM (4) and 1 mM (12), had ~50% kill rates for BSF trypanosomes at 2 h of incubation and were essentially 100% lethal at 4 h. DTT at 0.5 mM was less toxic, with ~80% and ~30% survival at 2 and 4 h, respectively, but the morphology of viable cells at the later time points was severely impacted (data not shown). Thapsigargin at a concentration (5 μM) known to inhibit the ER Ca²⁺ transporter in trypanosomes (27–30) completely blocked growth over 24 h, but lower concentrations (1 and 2.5 μM) had only a modest impact (Fig. 4A, center). Tunicamycin at 50 ng/ml was initially cytostatic and then cytotoxic over 24 h, but lower concentrations did not significantly affect growth (Fig. 4A, right).

Based on these results, we attempted to induce upregulation of BiP by acute treatment with the maximum concentrations of each

FIG 3 TbSec61α localization. In situ-tagged Ty-TbSec61α cells were fixed, permeabilized, and stained with anti-BiP (red) and anti-Ty (green). Kinetoplasts (k) and nuclei (n) were stained with DAPI (right panel). Deconvolved summed stack projections of single and triple merged channels and corresponding DIC images are presented.
of these compounds compatible with short-term viability: 0.5 mM DTT, 5 μM thapsigargin, and 200 ng/ml tunicamycin. In regard to tunicamycin, we have previously determined that this is the maximum concentration capable of complete inhibition of N-glycan synthesis in BSF trypanosomes without significant impact on protein synthesis and cell viability during short-term treatment (31). Cells were cultured with these reagents for 0 to 4 h, and cell extracts were analyzed for steady-state levels of BiP and VSG (Fig. 4B, top). Neither protein was affected by these treatments. Treated cells were also assayed for cytoplasmic Hsp70 (Fig. 4B, middle) and a second ER molecular chaperone, TbPDI2 (17) (Fig. 4B, bottom). Again, expression of these proteins was unaltered. Normalizing BiP expression to Hsp70 content over multiple biological replicates confirmed that no statistically significant increase in steady-state BiP levels resulted from any of these treatments (Fig. 4C). We conclude that trypanosomes are unable to generate a short-term response to the typical doses of pharmacological agents known to generate UPR in mammalian cells.

We next asked if more sustained subacute pharmacological treatment might result in a UPR-like response. For these experiments we focused on thapsigargin and tunicamycin because BSF trypanosomes are clearly not able to cope long-term with the harsh redox stress of DTT at any of the tested concentrations. Parasites were cultured with maximal sublethal concentrations of thapsigargin (Fig. 5A and C) or tunicamycin (Fig. 5B and D) for 24 h, and steady-state levels of BiP, VSG, Hsp70, and TbPDI2 were

FIG 4  Acute pharmacological treatment of trypanosomes. (A) BSF trypanosomes were harvested from log-phase cultures and subcultured in the presence of vehicle or the indicated concentrations of dithiothreitol (DTT), thapsigargin (TG), or tunicamycin (TN). Cell densities (means ± SEM, n = 3) were determined by hemocytometer. (B) Log-phase BSF trypanosomes were subcultured at 5 × 10^5 cells/ml in the presence of vehicle (Control), DTT (0.5 mM), TG (5 μM), or TN (200 ng/ml). At the indicated times cells were harvested, washed, and analyzed by SDS-PAGE and Western blotting. Samples were probed with rabbit anti-BiP (B) and rabbit anti-VSG (V) simultaneously, with rabbit anti-Hsp70, and with rabbit anti-TbPDI2 (αPDI2), as indicated. All lanes contain 2.5 × 10^5 cell equivalents based on initial cell densities; representative blots from the same experiment are shown. In each case full recovery of VSG and cytoplasmic Hsp70 at 4 h indicates equivalent loading of intact cells regardless of viability status. Stars indicate underglycosylated forms of newly synthesized VSG221 and TbPDI2 that accumulate during the incubation period. (C) BiP signals for control and DTT-, TG-, and TN-treated samples were quantified and normalized to the corresponding Hsp70 signals. Means ± SEM are presented for three biological replicates.

FIG 5  Subacute pharmacological treatment of trypanosomes. (A and B) Log-phase BSF trypanosomes were subcultured at 1 × 10^5 cells/ml in the indicated concentrations of thapsigargin (TG) or tunicamycin (TN) at 24 h cells were harvested, washed, and analyzed by SDS-PAGE and Western blotting. As indicated, samples were probed with anti-BiP (B) and anti-VSG (V) simultaneously, anti-Hsp70 (H), or anti-PDId2 (P). All lanes contain 2.5 × 10^5 viable cell equivalents; representative blots from the same experiment are shown for BiP and VSG, Hsp70, and TbPDI2. Stars indicate partially glycosylated forms of newly synthesized VSG221 and TbPDI2 that accumulate during long-term incubation with tunicamycin. (C and D) BiP signals for TG- and TN-treated samples were quantified and normalized to the corresponding Hsp70 signals (B/H). Means ± SEM are presented for three biological replicates.
assessed by Western blotting. Again, no significant effect of either agent, at any concentration, was detected on the steady-state levels of any of these endogenous reporters. To investigate the existence of a UPR-like response at the mRNA level, we performed qRT-PCR with cDNA prepared from cells following acute and subacute treatments with thapsigargin or tunicamycin. In no case was an alteration in the message levels of the ER chaperones BiP and TbPDI2 observed (Fig. 6A). Collectively these results suggest that pharmacological treatments intended to apply persistent ER stress do not elicit a UPR-like response in BSF trypanosomes.

**Pharmacological treatment does not induce an SLS response.** The lack of a UPR-like response does not rule out the possibility that an SLS response is induced by these treatments. Therefore, we used qRT-PCR to access the effect of pharmacological treatments on the levels of SL RNA and tSNAP42 mRNA (Fig. 6B). Again, neither acute nor subacute treatments induced the hallmark indicators of SLS, upregulation of tSNAP42 and loss of SL RNA. These results strongly indicate that spliced leader silencing does not occur in response to pharmacologically induced ER stress in BSF trypanosomes.

**DISCUSSION**

From the outset it must be noted that pharmacological and genetic characterization of SLS as a UPR-like response has been done primarily in PCF trypanosomes (4, 7, 8, 11), and this may contribute to some of the discrepancies with the work we present here, which is performed exclusively in BSF parasites. Nevertheless, two of the central stimuli used to elucidate these responses in PCF cells, disruption of the translocation machinery and DTT treatment, were applied to BSF trypanosomes with similar results, and this has been used to argue that SLS is not a stage-specific phenomenon (4). The effects of TbSec61α ablation are discussed below, but our results clearly indicate, within the limits imposed by issues of toxicity and cell viability, that none of the pharmacological agents commonly used in mammalian cells induce a UPR-like response in BSF trypanosomes, as judged by elevation in steady-state levels of the ER chaperones BiP and TbPDI2. This holds for both short-term acute and long-term subacute treatment regimens. Most importantly, we find that DTT at a level 1/10 that typically used to induce UPR in mammalian cells (0.5 versus 5.0 mM) is lethal to BSF trypanosomes, and consequently any conclusions based on this reagent must be treated with suspicion. In this regard, our results with DTT contradict those of Goldschmidt et al. (4) while confirming and extending those of Koumandou et al. (12). The latter investigators argued that gene expression within the replicative stages of the trypanosome life cycle is relatively refractory to extrinsic stimuli. Whether trypanosomes could mount a UPR-like response to a bona fide intrinsic ER stress, for instance accumulation of misfolded native secretory cargo, remains to be determined. However, tunicamycin is the pharmacological reagent most likely to provide such a stimulus. N-Glycans are critical for the folding of many secretory proteins (32), and tunicamycin is an effective inhibitor of N-glycosylation in BSF trypanosomes (31, 33, 34). Yet it has no effect on transport of VSG, the major secretory cargo of BSF trypanosomes, which represents 10% of all cellular protein (24) and proportionally a much higher percentage of total secretory flux through the ER. Tunicamycin does inhibit the forward trafficking of other highly glycosylated secretory cargo, such as the lysosomal membrane protein p67 (C. Tiengwe and J. D. Bangs, unpublished observations), but based on our current results, this is apparently not enough to induce a UPR-like response in BSF trypanosomes. Our findings with tunicamycin are consistent with those of Koumandou et al. (12) and Izquierdo et al. (38), both of whom found no induction of BiP. The latter authors also found that neither heat shock, nor knocking out a component of the ER folding machinery (UDP-glucose:glycoprotein glycosyltransferase), affected BiP expression. Both groups also concluded that UPR-like responses do not occur in BSF trypanosomes.

It is a separate question as to whether these pharmacological treatments induce an SLS response, as defined by upregulation of tSNAP42 and loss of SL RNA, and if so whether it is truly related to persistent ER stress. The SLS response was originally demonstrated by ablation of selected factors that affect import of nascent secretory proteins into the ER (TbSRα, TbSec61α, and TbSec63) (7). It was implied that disrupting translocation somehow translates into internal stress in the ER. However, our results indicate that when the translocation machinery is ablated, BSF cell growth ceases at a point where full translocation competence is maintained (discussed below). Consequently, there can be no accumulation of misfolded secretory proteins in the cytoplasm, and the proteins that are imported are glycosylated, processed, and transported normally. In light of these results, it is difficult to see how ablating the translocation machinery constitutes a legitimate internal ER stress. Thus, the only connection between persistent ER stress and the SLS response is the claim that DTT treatment has the same effect as RNAi targeting the aforementioned translocation factors (4, 7, 8, 11). Our work here disqualifies DTT, and in our opinion, as with the UPR-like response, any conclusions about SL silencing based on use of this reagent should be regarded with...
extreme caution. However, other extrinsic stresses, deoxyglucose and acidic culture (pH 5.5, 2 to 3 days), also apparently induce SLS in PCF trypanosomes (4, 6). Might these represent true ER stress? The action of deoxyglucose is thought to be via inhibition of N-glycan synthesis; but deoxyglucose is a potent inhibitor of glycolysis, and the effects of this compound are likely to be highly pleiotropic. It should be noted that deoxyglucose cannot be used with BSF trypanosomes as they are dependent solely on glycolysis for energy metabolism. Likewise, culture at low pH, which we would argue is a global cellular stress rather than a specific ER stress, cannot be attempted with BSF trypanosomes as they are unable to cope with prolonged acidic conditions. We have, however, investigated the ability of acute and subacute treatments with thapsigargin and tunicamycin to induce an SLS response. Neither agent, each of which directly affects critical ER functions and therefore could legitimately result in persistent ER stress, led to the hallmark indicators of SLS in BSF trypanosomes. The fact that tunicamycin, a direct and specific inhibitor of N-glycan synthesis, does not induce an SLS response in BSF trypanosomes lends support to the notion that, whatever effect deoxyglucose has on PCF trypanosomes, it does not directly related to secretory protein glycosylation in the ER.

Our finding that TbSec61α silencing does not affect translocation was unexpected. Assuming that it is a relatively stable protein, TbSec61α must be lost during RNAi silencing by dilution among daughter cells. Once the per-cell content reaches a critically low level (~16 h), cell growth ceases even though enough functional translocons remain to accommodate all newly synthesized secretory proteins. This fine balance is maintained temporarily, but ultimately (~24 h) cell death ensues, indicating that cell division is closely tied to translocation capacity. This situation is different from that of growth arrest following silencing of the major secretory cargo, VSG (35, 36). VSG silencing results in precise precytokinesis arrest with global shutdown of protein translation, whereas TbSec61α silencing does not affect cell cycle profile or translation, suggesting that trypanosomes have distinct mechanisms for monitoring VSG versus general secretory cargo.

In summary, our results rule out any connection between pharmacological ER stress or disruption of the secretory translocation machinery and the induction of UPR-like and SLS responses in BSF trypanosomes. Our current work does not rule out that the situation is different in PCF cells, but if so, such responses are clearly stage-specific phenomena. More importantly, it remains to be seen whether a bona fide intrinsic ER stress, such as an overexpressed misfolded secretory protein, can induce either UPR-like or SLS responses.

ACKNOWLEDGMENTS

We are grateful to Derek Nolan (Trinity College, Dublin, Ireland) for the generous gift of anti-TbPDD12. This work was supported by U.S. Public Health Service grant R01 AI035739 to J.D.B.

REFERENCES

23. Osborne AR, Rapoport TA, van den Berg B. 2005. Protein translocation


