Phosphorylation of the TATA-binding protein activates the spliced leader silencing pathway in *Trypanosoma brucei*

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PARASITOLOGY

Phosphorylation of the TATA-binding protein activates the spliced leader silencing pathway in *Trypanosoma brucei*

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The parasite *Trypanosoma brucei* is the causative agent of human African sleeping sickness. *T. brucei* genes are constitutively transcribed in polycistronic units that are processed by trans-splicing and polyadenylation. All mRNAs are trans-spliced to generate mRNAs with a common 5′ exon derived from the spliced leader RNA (*SL RNA*). Persistent endoplasmic reticulum (ER) stress induces the spliced leader silencing (SLS) pathway, which inhibits trans-splicing by silencing *SL RNA* transcription, and correlates with increased programmed cell death. We found that during ER stress induced by *SEC63* silencing or low pH, the serine-threonine kinase PK3 translocated from the ER to the nucleus, where it phosphorylated the TATA-binding protein TRF4, leading to the dissociation of the transcription preinitiation complex from the promoter of the *SL RNA* encoding gene. PK3 loss of function attenuated programmed cell death induced by ER stress, suggesting that SLS may contribute to the activation of programmed cell death.

INTRODUCTION

Trypanosomes and related organisms are parasitic protozoa causing infamous diseases such as African sleeping sickness (*Trypanosoma brucei*), leishmaniasis (*Leishmania* spp.), and Chagas' disease (*Trypanosoma cruzi*). Trypanosomes use a trans-splicing mechanism for the maturation of mRNAs, in which a common exon from a small nuclear RNA (snRNA) known as the spliced leader RNA (*SL RNA*) is spliced onto the 5′ end of other mRNAs. The multicopy *SL RNA* gene is the only gene in *T. brucei* that has a defined RNA polymerase II (pol II)–binding promoter and transcriptional start site. The promoter consists of a bipartite upstream sequence element (USE) and an initiator element at the transcription start site (*1*–*4*), and a conventional, albeit divergent, preinitiation complex drives transcription of the *SL RNA* gene. Transcription of *SL RNA* requires the snRNA-activating protein complex (SNAPc) composed of SNAP50 (also known as tSNAP50), SNAP2 (tSNAP42), and SNAP3 (tSNAP26) (*5*, *6*). tSNAPc binds to the USE, likely through SNAP2, which contains a Myb DNA binding domain. tSNAPc is part of a larger protein complex that also comprises trypanosome homologs of TATA-binding protein (TBP), termed TBP-related factor 4 (TRF4), and transcription factor IIA (TFIIA) (*5*, *6*). Moreover, TRF4 (*7*), TFIIB (*8*, *9*), TFIIH (*10*, *11*), putative TFIIE homologs TSP1 and TSP2 (*12*), and the Mediator complex (*13*) are all required for *SL RNA* transcription.

Because regulation of gene expression in trypanosomes is primarily posttranscriptional (*14*), they lack the transcription-mediated mechanism of the unfolded protein response (UPR), which, in other eukaryotes, promotes the transcription of genes encoding proteins such as chaperones that can help cope with the stress (*15*). Instead, trypanosomes respond to endoplasmic reticulum (ER) stress by stabilizing mRNAs essential for survival during persistent stress (*16*).

Under persistent ER stress, *T. brucei* inhibit gene expression by a unique mechanism that we termed spliced leader silencing (SLS) because it occurs through silencing of *SL RNA* expression (*16*, *17*). ER stress can be induced experimentally by silencing genes encoding the signal recognition particle (SRP) receptor (SRα) or proteins such as SEC61, the translocation channel, or SEC63, a protein essential for both post- and cotranslational protein translocation (*18*). Thus, for SLS to occur in response to ER stress, a signal must be transmitted from the ER to the nucleus to inhibit *SL RNA* transcription, and thereby disrupt trans-splicing and prevent maturation of all mRNAs. SLS is characterized by the reduced abundance of *SL RNA* and increased abundance of SNAP2. SNAP2 normally localizes to discrete punctae within the nucleus associated with sites of *SL RNA* synthesis. However, during SLS, SNAP2 localizes throughout the nucleus because it fails to bind the *SL RNA* promoter (*16*, *17*).

Induction of SLS correlates with programmed cell death (PCD), manifested by appearance of phosphatidyl serine on the cell surface, DNA laddering, chromatin condensation, increased reactive oxygen species and cytoplasmic Ca^{2+}, and decreased mitochondrial membrane potential (*16*). Accordingly, and despite the lack of caspases in trypanosomes (*19*), we proposed that SLS may trigger a PCD-like death pathway (*16*). Thus, SLS may have evolved as a mechanism for the removal of unfit parasites from the population ensuring successful host infection (*20*).

The trypanosome proteome contains homologs of eukaryotic initiation factor 2 (eIF2) kinases, which play a role in the UPR in other organisms. In mammals, the ER-localized transmembrane eIF2α kinase PERK (protein kinase RNA–like ER kinase) is activated during the UPR, which inhibits translation and further accumulation of misfolded proteins (*21*). The *T. brucei* eIF2α homolog TbeIF2K2 has a transmembrane domain but is probably not a functional homolog of PERK because it localizes to the flagellar pocket, not the ER, where it functions in endocytosis (*22*). The *Leishmania* homolog of PERK localizes to the ER where it functions in the differentiation process from the insect stage (promastigote) to the form that propagates in the mammalian host (amastigote) (*23*) and is phosphorylated during the UPR (*24*). The *T. cruzi* PERK homolog is required for the differentiation from the epimastigote to the infective metacyclic form and is activated by starvation (*25*). In *T. brucei,* another eIF2 paralog, TbeIF2K3 (herein referred to as PK3), has no known function and contains an unusual insert located between the VIB and VII motifs in the kinase domain (*22*). Thus, serine-threonine kinases related to eIF2 kinases have diverse functions in

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the different trypanosomatid species and may play a role in ER stress–induced SLS.

Here, we revealed the mechanism underlying ER stress–induced SLS by identifying the key signaling event that connects ER stress to *SL RNA* silencing in the nucleus. Purification and mass spectrometry (MS)–based analysis of the *SL RNA* preinitiation transcription complex in trypanosomes with *SEC63* silencing revealed that TRF4 is phosphorylated on Ser^{35} during ER stress, and mutant $TRF4^{S35Q}$ did not detach from the promoter of *SL RNA* during ER stress. Moreover, PK3 was phosphorylated, translocated from the ER to the nucleus, and copurified with the *SL RNA* transcription complex during *SEC63* silencing–induced ER stress. Silencing of *PK3* prevented phosphorylation of TRF4, SLS, and PCD. Overall, our results support a model in which persistent ER stress induced by *SEC63* silencing or low pH induces PK3 activity, causing dissociation of TRF4 from the *SL RNA* transcription preinitiation complex leading to SLS and PCD.

RESULTS

The *SL RNA* transcription complex is disrupted by ER stress

We previously found that ER stress induced by low pH, reducing agents, or depletion of SRα or SEC63 causes increased abundance of SNAP2 and changes the localization of SNAP2 from the site of *SL RNA* transcription to a diffuse distribution throughout the nucleus (*16*, *17*). To examine whether other proteins involved in the transcription of *SL RNA* also change abundance and localization in trypanosomes exposed to ER stress, we stably expressed PTP-tagged TRF4, TFIIA2, or SNAP3 in *T. brucei* containing a tetracycline-inducible vector expressing a stem loop construct that promotes the degradation of *SEC63* mRNA (*18*). Similar to SNAP2, the abundance of TRF4, TFIIA2, and SNAP3 was greater in cells exposed to tetracycline compared to those that were not (Fig. 1A). Moreover, PTP-tagged TRF4, TFIIA1, TFIIA2, SNAP3, and SNAP50 showed diffuse nuclear localization in cells with *SEC63* silencing (Fig. 1B). Thus, silencing of *SEC63* caused the dissociation of the transcription preinitiation complex from the *SL RNA* promoter.

MS analysis reveals that TRF4 interacts with PK3 and is phosphorylated during ER stress

To identify the underlying mechanism of activation of the SLS pathway, we examined changes in the composition of the *SL RNA* transcription preinitiation complex during ER stress. We performed tandem affinity purification from lysates of cells stably expressing PTP-tagged TRF4, SNAP2, or SNAP3 incubated with or without tetracycline to induce *SEC63* silencing. MS analysis of the affinity-purified complexes from TRF4- and SNAP2-overexpressing cells identified members of the *SL RNA* transcription preinitiation complex (*7*, *8*), including SNAP50, SNAP2, TRF4, TFIIA1, and TFIIA2, in the presence and absence of *SEC63* silencing (Fig. 2A and table S1). In addition, we discovered that Ser^{35} of TRF4 was phosphorylated, and that PK3 copurified with TRF4 specifically in

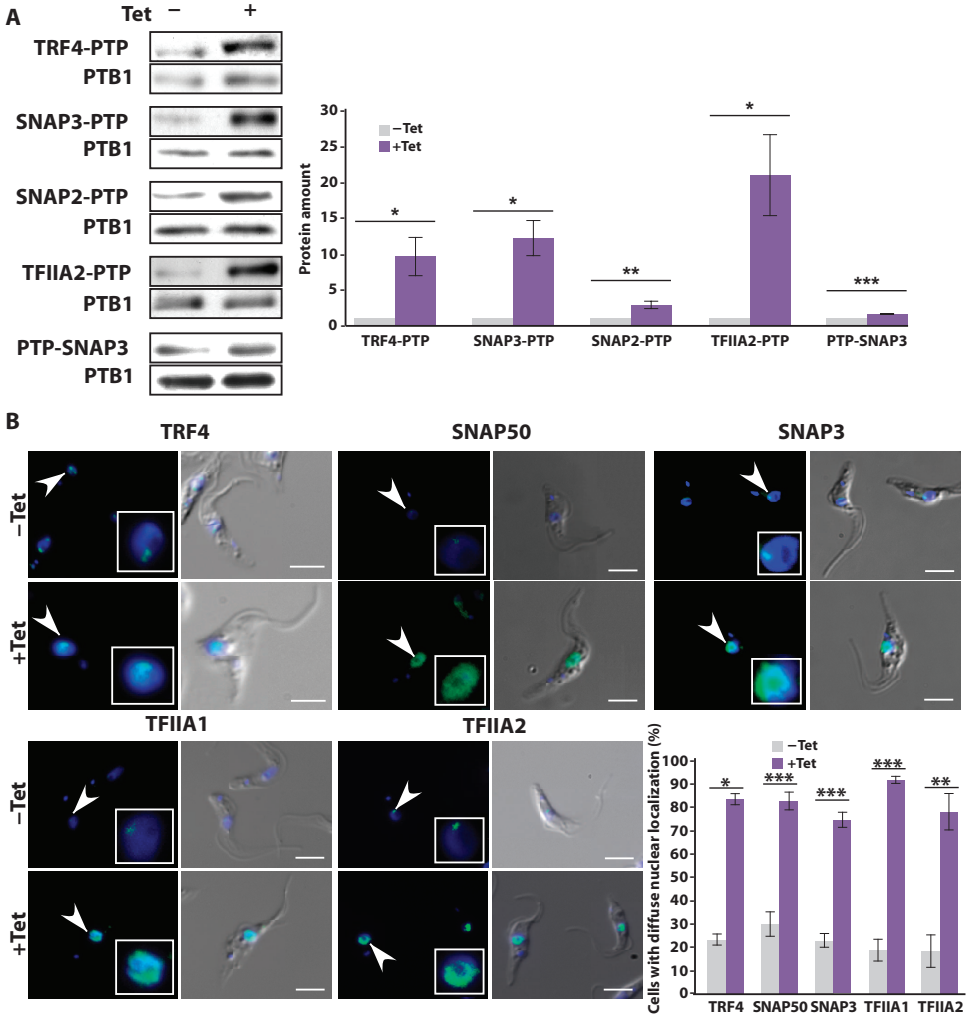

Fig. 1. Disruption of the *SL RNA* transcription complex is induced by ER stress. (A and B) Cells stably expressing the indicated PTP-tagged proteins and a tetracycline-inducible stem-loop construct targeting *SEC63*. *SEC63* silencing was either not induced (–Tet) or induced with tetracycline (+Tet) for 2.5 days. (A) Western blots from nuclear lysates. An antibody against IgG was used to detect the PTP tag, and PTB1 immunoreactivity was used as a loading control. The graph represents the mean ± SEM of blots from three independent experiments. Protein abundance was normalized to PTB1 and presented as fold change relative to –Tet. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ compared to –Tet, one-sample *t* test. (B) Immunofluorescence labeling of cells with 4′,6-diamidino-2-phenylindole (DAPI; blue) to label nuclei and antibodies against IgG to recognize the PTP tag (green). Panels on the right are differential interference contrast (DIC) images. Enlargements of the nuclear area are shown as insets. Scale bars, 5 μm. The graph represents the mean ± SEM of quantification from three independent experiments with more than 95 cells per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ compared to –Tet, Student's *t* test.

SEC63-silenced cells (table S1). We also found that PK3 was phosphorylated in *SEC63*-silenced cells (table S1). Thus, these changes in protein interaction and posttranslational modification could be important for activation of the SLS pathway.

To confirm that the phosphorylation of TRF4 identified by MS was induced in cells with ER stress, we mutated Ser^{35} to Glu in TRF4 ($TRF4^{S35Q}$) and created stable cell lines. Immunoblotting indicated an increase in apparent molecular weight of TRF4, but not $TRF4^{S35Q}$, in cells with *SEC63* silencing (Fig. 2B).

To assess the possibility that modification of TRF4 was specific to ER stress induced by silencing of *SEC63*, we examined TRF4 in cells exposed to low pH, which induces ER stress and SLS (*17*). We found that the apparent molecular weight of TRF4 was increased at pH 5.5 compared to pH 7.0 (Fig. 2C). Thus, TRF4 is posttranslationally modified at a single residue (Ser^{35}), likely by phosphorylation, under conditions that induce ER stress.

Phosphorylation of TRF4 during ER stress causes dissociation from the promoter of the *SL RNA* encoding gene

To examine if the posttranslational modification of TRF4 was required for it to detach from the *SL RNA* transcription site during ER stress, we monitored the localization of TRF4 or $TRF4^{S35Q}$ N-terminally tagged with yellow fluorescent protein (YFP) in cells carrying the *SEC63* silencing construct. In cells that were not silenced, both YFP-TRF4 and YFP-$TRF4^{S35Q}$ localized in one or two distinct perinucleolar spots (Fig. 3A), consistent with the localization sites of *SL RNA* transcription (*16*). In cells with *SEC63* silencing, YFP-TRF4 occupied the entire nucleus, whereas YFP-$TRF4^{S35Q}$ remained in the sites of *SL RNA* transcription (Fig. 3A), suggesting that the modification of Ser^{35} promotes detachment of TRF4 from the *SL RNA* transcription site. Moreover, as in cells with *SEC63* silencing, TRF4 had diffuse nuclear localization in cells exposed to low pH (fig. S1).

We also assessed whether the posttranslational modification of TRF4 that led to a change in its localization resulted in a loss of the interaction between TRF4 and the promoter of the *SL RNA* encoding gene. Using chromatin immunoprecipitation (ChIP) with a ChIP-grade antibody directed against green fluorescent protein, which also recognizes YFP, we found that *SEC63* silencing reduced the binding of YFP-TRF4, but not TRF4-YFP^{S35Q}, to a region of the proximal promoter of *SL RNA* (Fig. 3B). We confirmed the specificity of YFP-TRF4 ChIP by amplification of a negative control region of a gene encoding ribosomal RNA (Fig. 3B). Thus, posttranslational modification of TRF4 on Ser^{35} during ER stress likely inhibits binding to the promoter of the *SL RNA* gene, leading to diffusion of TRF4 throughout the nucleus.

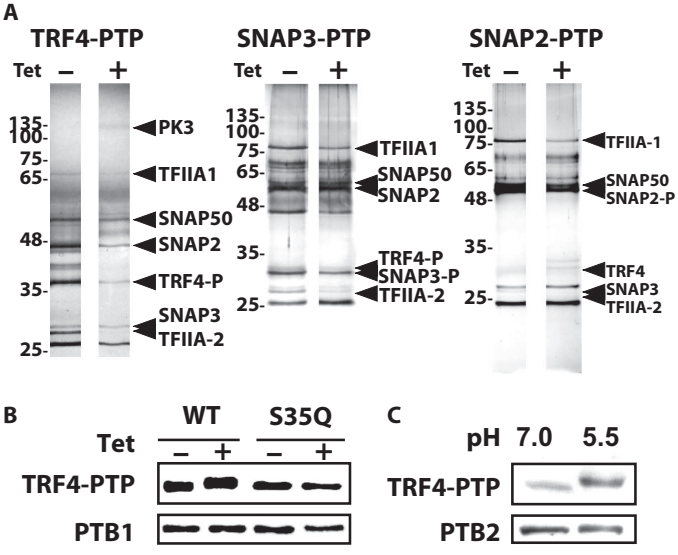

Fig. 2. ER stress alters the composition of the *SL RNA* preinitiation transcription complex and modification of TRF4. (**A**) Silver-stained protein gels of proteins purified from cells expressing the indicated PTP-tagged proteins and the *SEC63* silencing construct without (–) or with (+) tetracycline-induced *SEC63* silencing for 2.5 days. The molecular mass markers and the identity of the proteins are indicated. Data are representative of at least two experiments for each purification. (**B**) Western blot of nuclear lysates from cells expressing TRF4-PTP or $TRF4^{S35Q}$-PTP and the *SEC63* silencing construct. *SEC63* silencing was either not induced (–) or induced (+) for 2.5 days. PTB1 was used as a loading control. WT, wild type. (**C**) Western blot of nuclear lysates from cells expressing TRF4-PTP and exposed to media with the indicated pH for 3 days. PTB2 was used as a loading control. For (B) and (C), the blots are representative of $n = 3$ independent experiments.

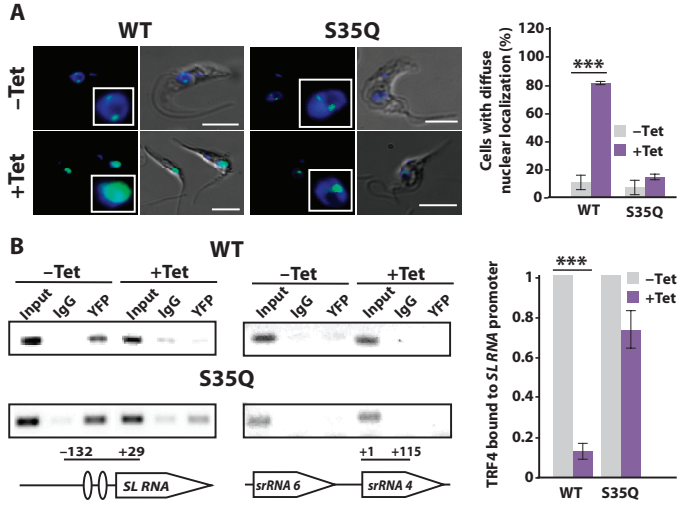

Fig. 3. Ser^{35} of TRF4 is required for its dissociation from the *SL RNA* transcription preinitiation complex during ER stress. (**A** and **B**) Cells expressing YFP-TRF4 or $TRF4^{S35Q}$-YFP and the *SEC63* silencing construct. (A) Fluorescence imaging of cells without (–Tet) or with (+Tet) *SEC63* silencing for 2 days. DAPI (blue) was used to label nuclei, and YFP (green) indicates the tagged protein. Panels on the right are DIC images. Enlargements of the nuclear area are shown as insets. Scale bars, 5 μm. The graph represents the mean ± SEM of quantification from three independent experiments with more than 95 cells per condition. $^{***}P < 0.005$ compared to –Tet, Student's *t* test. (B) DNA gels showing the indicated amplicons from ChIP analysis. ChIP was performed using a negative control antibody (IgG) or an antibody that recognizes YFP on DNA from cells without (–Tet) or with (+Tet) *SEC63* silencing for 2.5 days. The input and pellet DNA were diluted 1:800 and 1:20, respectively. Nucleotides are numbered with respect to the transcription start site. The graph represents the mean ± SEM of quantification from three independent experiments. $^{***}P < 0.005$ compared to –Tet, one-sample *t* test. The amount *SL RNA* DNA that coprecipitated with the YFP antibody in +Tet cells was quantified relative to that in –Tet cells using the input signal for normalization.

PK3 is essential for SLS

Our MS analysis showed that PK3 copurified with TRF4 in cells with *SEC63* silencing; thus, we speculated that PK3 could phosphorylate TRF4, leading to its dissociation from the promoter of the *SL RNA* gene, thus suggesting that PK3 is essential for activation of SLS. We generated a stable cell line with tetracycline-inducible constructs for silencing both *PK3* and *SEC63* and with PTP-tagged PK3, and found that exposing these cells to tetracycline reduced the abundance of PK3-PTP within 2 days (Fig. 4A).

Because the activation of kinases is often associated with phosphorylation of the kinase and because our MS analysis identified phosphorylation of PK3 and TRF4 in cells with *SEC63* silencing, we asked whether PK3 was required for the phosphorylation of TRF4. Western blot analysis revealed that the apparent molecular weight of PK3-PTP was increased in cells with *SEC63* silencing (Fig. 4B), consistent with phosphorylation of PK3 during ER stress. Moreover, the increase in the apparent molecular weight of PTP-TRF4 caused by *SEC63* silencing was abolished in cells with silencing of both *SEC63* and *PK3* (Fig. 4C). Silencing of *PK3* also abolished the putative posttranslational modification of TRF4 in cells exposed to low pH (Fig. 4D). We performed in vitro phosphorylation reactions using PTP-TRF4 and PTP-PK3 purified from trypanosomes and found that although PTP-PK3 could undergo autophosphorylation in vitro, it was not able to phosphorylate TRF4 under these conditions (fig. S2). Thus, we speculated that either an additional protein was required to promote the phosphorylation of TRF4 by PK3 or the presence of a phosphatase associated with the purified proteins removed the phosphate from TRF4. We also asked whether loss of PK3 affected the localization of TRF4 and found that PTP-TRF4, which was diffuse throughout the nucleus in cells with *SEC63* silencing, localized to *SL RNA* transcription sites in cells silenced for both *SEC63* and *PK3* (Fig. 4E).

To determine if PK3 was required for SLS, we examined the abundance of *SL RNA* in cells with *SEC63* and *PK3* silencing. Whereas silencing of *SEC63* alone decreased the amount of *SL RNA* within 3 days, silencing of both *SEC63* and *PK3* had no effect on the amount of *SL RNA* for up to 4 days (Fig. 4F). To control for off-target effects of the *PK3* silencing construct, we repeated the experiment using a second nonoverlapping sequence to silence *PK3* and found similar results (Fig. 4G). To verify that silencing *PK3* did not inhibit silencing of *SEC63*, we measured the amount of the glycosylphosphatidylinositol-anchored procyclin EP, which is reduced in trypanosomes that are deficient for SEC63 (*18*). Cells with either *SEC63* silencing or *SEC63* and *PK3* silencing (fig. S3) had a significant reduction in the abundance of EP abundance (fig. S3), suggesting that protein translocation defects that occur with loss of SEC63 were not affected by loss of PK3 and that, by inhibiting PK3, it is possible to uncouple protein translocation defects from the induction of SLS. Thus, collectively, these data suggest that during ER stress, PK3 phosphorylates TRF4, causing it to dissociate from the promoter of the *SL RNA* gene, resulting in SLS.

PK3 relocalizes from the ER to the nucleus during ER stress

Because we found that PK3 was essential for SLS, we examined whether it was responsible for the transmission of the ER stress signal from the ER to the nucleus. We examined the localization of PK3 in cells stably expressing PK3-YFP. In the absence of *SEC63* silencing, PK3-YFP partially colocalized with the ER-resident chaperone protein BiP and with SRP19, a subunit of SRPs bound to the surface of the ER (*26*), whereas in cells with *SEC63* silencing, a fraction of PK3-YFP translocated to the nucleus (Fig. 5, A and B). Biochemical fractionation and Western blot analysis confirmed that the abundance of PK3 was increased in the nucleus of cells with *SEC63* silencing (Fig. 5C). Finally, we used immunogold-based transmission electron microscopy for the PTP epitope in cells expressing PTP-PK3 and found that, under basal conditions, PTP-PK3 localized in a perinuclear distribution,

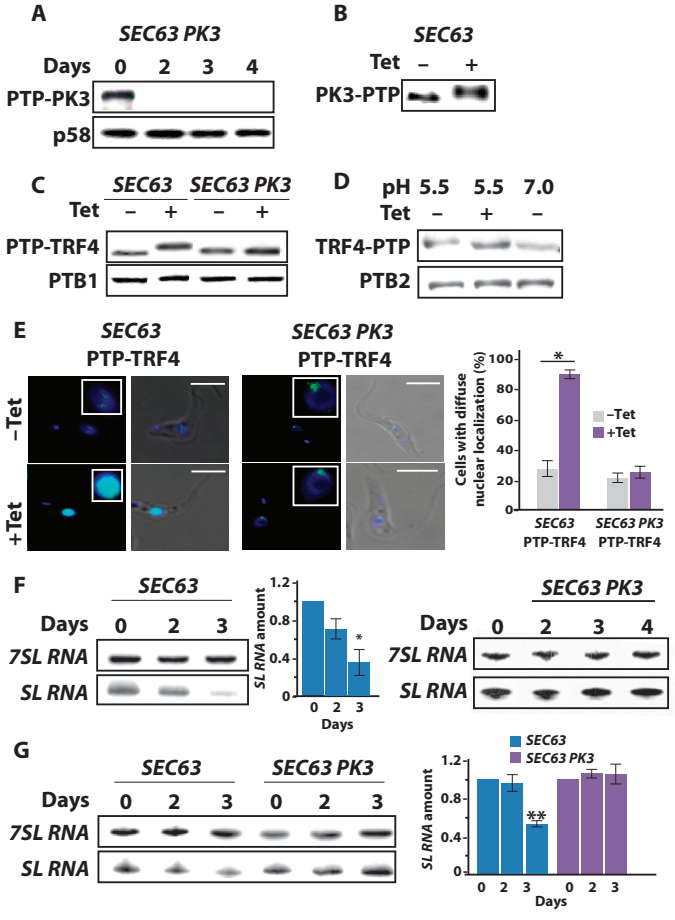

Fig. 4. PK3 is required for the posttranslational modification of TRF4 and induction of SLS during ER stress. (A to D) Western blots of lysates from cells expressing (A) PTP-PK3 and the *SEC63* and *PK3* silencing constructs with silencing induced with tetracycline for the indicated number of days, or (B) PK3-PTP and the *SEC63* silencing construct, (C) PTP-TRF4 and the *SEC63* or *SEC63* and *PK3* silencing constructs, or (D) TRF4-PTP and the *PK3* silencing constructs with (+Tet) or without (–Tet) silencing for 2.5 days. Cells in (D) were incubated in media with the indicated pH for three additional days in the presence or absence of tetracycline. PTB1, PTB2, or p58 was used as a loading control. Blots are representative of three independent experiments. (E) Immunofluorescence labeling of cells expressing PTP-TRF4 and the *SEC63* or *SEC63* and *PK3* silencing constructs with (–Tet) or without (–Tet) silencing for 2 days. DAPI (blue) was used to label nuclei, and antibodies against IgG were used to recognize the PTP tag (green). Panels on the right are DIC images. Enlargements of the nuclear area are shown as insets. Scale bars, 5 μm. The graph represents the mean ± SEM of quantification from three independent experiments with more than 95 cells per condition. $^{*}P < 0.05$ compared to –Tet, Student's *t* test. (F and G) Northern blots of total RNA from the cells expressing PK3-PTP and *SEC63* or *PK3* and *SEC63* silencing constructs and silenced for the indicated number of days. Blots were probed for *SL RNA*, or *7SL RNA* as a loading control. The cells used in (G) expressed a *PK3* silencing construct targeting a different sequence (nucleotides 609 to 1131) from that used in (F) (table S2). For (F) and (G), the graphs represent mean ± SEM of quantification from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to –Tet, one-sample *t* test.

resembling other ER-associated proteins, whereas in cells with *SEC63* silencing, PK3 could be found in the nucleus (Fig. 5D). Because PK3 lacks an obvious transmembrane domain, these data support a model in which PK3 is localized on the surface of the ER, and during ER stress, a subset of PK3 translocates to the nucleus, consistent with the model that PK3 interacts with the nuclear protein TRF4 and inhibits *SL RNA* transcription.

PK3 is essential for ER stress–induced PCD

Our discovery that PK3 was an essential enzyme in the SLS pathway enabled us to evaluate the role of SLS in triggering PCD. Unlike control cells, cells exposed to tetracycline to induce *SEC63* silencing displayed a broad hypodiploid (sub-G_1) peak when permeabilized with ethanol and stained with propidium iodide (PI), indicative of DNA fragmentation (Fig. 6A). To further evaluate the type of cell death, we labeled nonpermeabilized cells with the PS-binding protein annexin V to identify cells in early stages of PCD and counterstained them with PI, which only enters cells with plasma membrane degradation indicative of late stages of PCD. As expected, silencing of *SEC63* increased both the percentage of annexin V–positive and annexin V and PI double-positive cells (Fig. 6B). Moreover, when we examined cells with silencing of both *SEC63* and *PK3*, we found a significant reduction in the percentage of cells with DNA fragmentation (Fig. 6A) and an inhibition or delay in the progression of PCD (Fig. 6B) compared to cells with *SEC63* silencing alone. Thus, these data indicate that PK3-dependent signaling, likely through the SLS pathway, is required for efficient activation of PCD by ER stress.

DISCUSSION

SLS is activated by persistent ER stress induced by pH changes or perturbation of protein translocation to the ER, which is achieved experimentally by depleting ER translocon proteins (*16*–*18*). Here, we found that silencing *SEC63* increased the amounts of all the preinitiation transcription factors analyzed, suggesting that decreased *SL RNA* transcription leads to decreased turnover or increased synthesis of these proteins. In addition, we found evidence that SLS signaling involves the translocation of the ER-associated kinase PK3 to the nucleus, where it elicits the phosphorylation of the basal transcription factor TRF4. This in turn leads to dissociation of the RNA pol II transcription preinitiation complex from the promoter of the *SL RNA* gene, which enhances ER stress–induced PCD.

We found that *T. brucei* PK3 can act as a sensor of ER stress. Analysis of protein homology suggests that PK3 is a serine-threonine kinase of the eIF2α kinase family (*22*); however, PK3 lacks the transmembrane domain present in TbeIF2K2. Unlike in many eukaryotes, heat shock, does not promote phosphorylation of TbeIF2α in *T. brucei* (*28*). However, ER stress does promote phosphorylation of eIF2α paralogs in *Leishmania infantum* and *Leishmania donavani* (*23*, *24*), and an eIF2α paralog is phosphorylated during differentiation of *T. cruzi* (*25*), suggesting that the ER stress response and its regulation may differ among trypanosomatid species. So far, there is no evidence for the existence of ER stress–induced SLS in *Leishmania*, and changes in pH that induce ER stress and SLS in *T. brucei* (*17*) are cues for differentiation in *Leishmania (29)*. Moreover, Ser^{35} of TRF4, which we found was modified in *T. brucei* with *SEC63* silencing in a PK3-dependent manner, is not conserved in other trypanosomatid species (*7*). Thus, SLS may be specific to *T. brucei*, similar to other cellular processes, such as the RNA interference pathway, which exist in *T. brucei* but not in most of the *Leishmania* species nor *T. cruzi* (*30*).

Our data do not show how ER stress activates PK3, but we hypothesize that this occurs through the interaction of PK3 with another ER-associated protein. PK3 may directly associate with the ER translocon. Changes in the

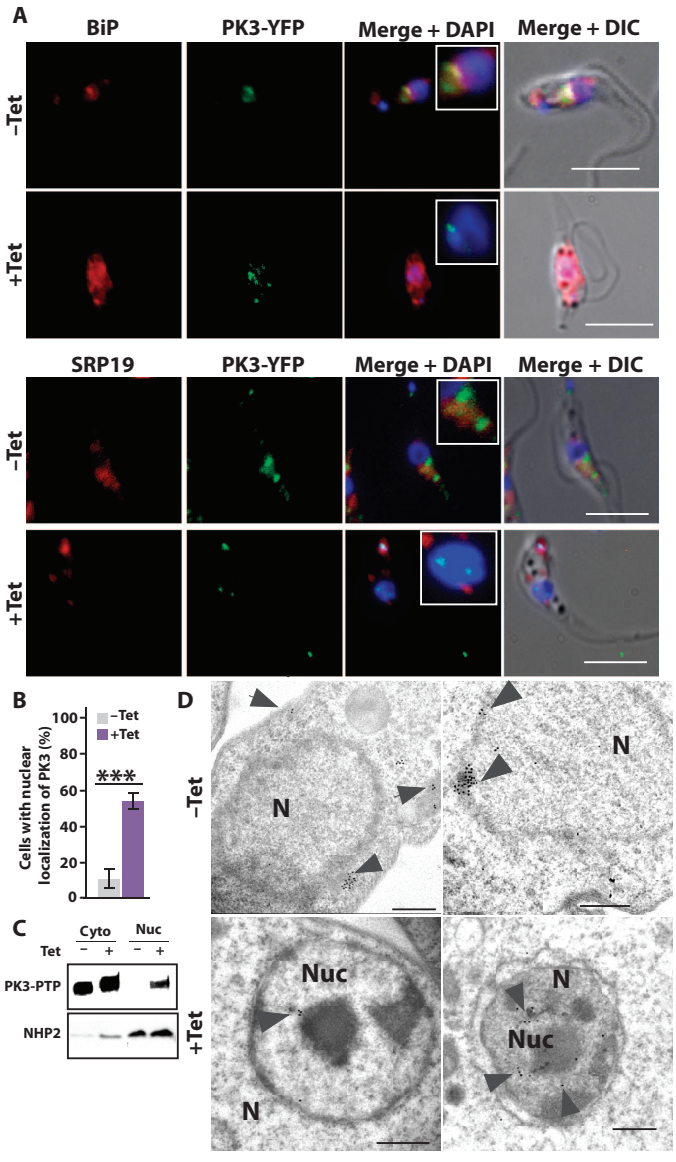

Fig. 5. PK3 translocates from the ER to the nucleus during ER stress. (A) Fluorescence imaging of cells expressing PK3-YFP and the *SEC63* silencing construct without (–Tet) or with (+Tet) *SEC63* silencing for 2 days. DAPI (blue) was used to label nuclei, YFP (green) indicates PK3, and BiP or SRP19 labels the ER (red). Enlargements of the nuclear area are shown as insets. Panels on the right are DIC images. Scale bars, 5 μm. (**B**) The graph represents the mean ± SEM of quantification of cells from (A) with overlapping PK3-YFP and DAPI staining from three independent experiments with more than 95 cells per condition. ***$P < 0.005$, Student's *t* test. (**C** and **D**) Cells expressing PK3-PTP and the *SEC63* silencing construct without (–Tet) or with (+Tet) *SEC63* silencing for 2.5 days. (C) Western blots of nuclear or cytoplasmic lysates. NHP2 (*27*) is used to control for the quality of fractionation. Blots are representative of three independent experiments. (D) Transmission electron microscopy of sections labeled with IgG antibodies to recognize the PTP tag. Representative images from two independent biological experiments and four technical replicates are shown. Arrowheads indicate the position of PK3-PTP. N, nucleus; Nuc, nucleolus. Scale bars, 0.5 μm.

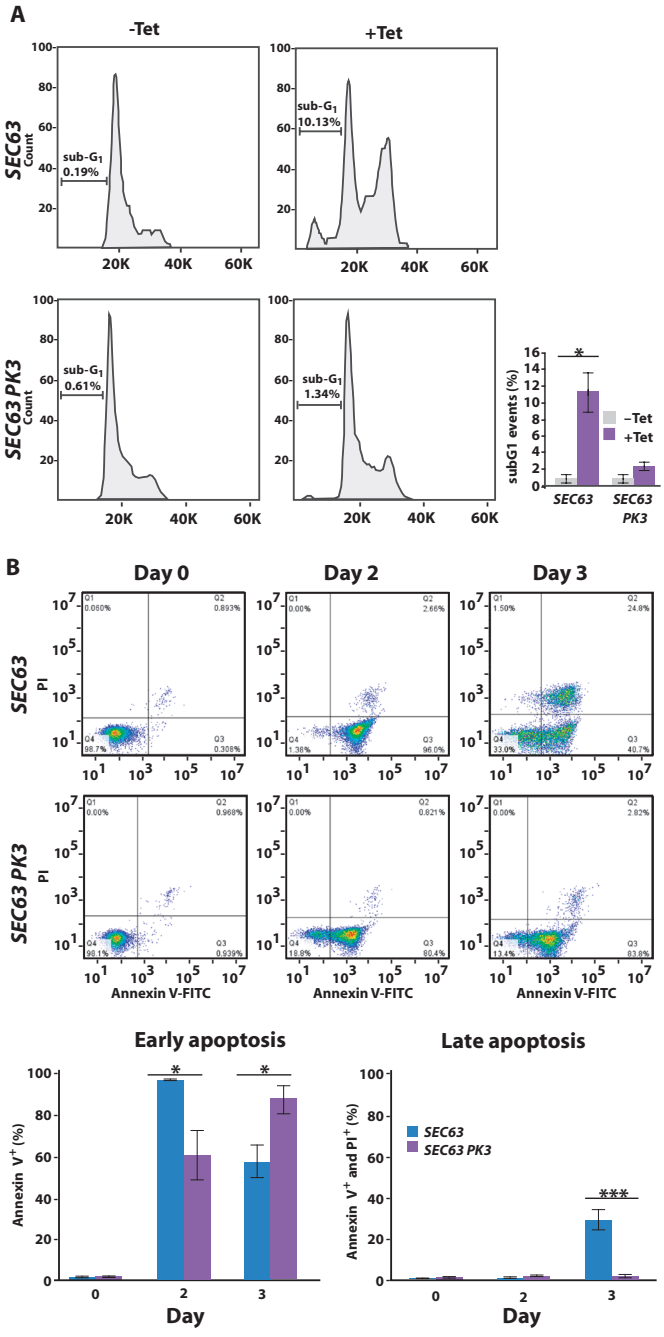

Fig. 6. PK3 is required for ER stress–induced PCD. (A and B) Cells expressing the *SEC63* or *SEC63* and *PK3* silencing constructs. (A) DNA content analysis of cells with (+Tet) or without (–Tet) silencing for 2.5 days. Cells were permeabilized and stained with PI and analyzed by flow cytometry. Graph represents the mean ± SEM of the percentage of cells with sub-G_1 DNA content quantified from three independent experiments. (B) Scatterplots of nonpermeabilized cells with silencing for the indicated number of days, stained with annexin V and PI, and analyzed by flow cytometry. Graphs represent the mean ± SEM of the percentage of cells that were annexin V–positive (early apoptosis) or annexin V– and PI-positive (late apoptosis) quantified from three independent experiments. For (A) and (B), $^*P < 0.05$ and $^{***}P < 0.005$, Student's *t* test.

translocon due to depletion of SEC63, SEC61, or SRα may trigger the release of PK3 from the ER, leading to its activation and translocation to the nucleus. Determining whether SLS signaling involves the activation of additional kinases or other proteins will require the identification of PK3-interacting proteins under normal conditions and during ER stress. Similarly, we did not determine if PK3 activation is sufficient to trigger SLS, or whether this pathway requires additional signaling steps. PK3 does not have an obvious nuclear localization signal, and thus, its translocation may require interaction with importins, as was shown, for example, for mitogen-activated protein kinases (*31*).

Similar to our observation that the *SL RNA* transcription complex disassembles during SLS in *T. brucei*, the promoter-binding transcription initiation complex of genes encoding snRNAs disassembles during M phase in mammalian cells (*32*). Mammalian U-rich snRNAs are synthesized by RNA pol II, with the exception of *U6* snRNA, which is synthesized by RNA pol III. Both RNA pol II– and RNA pol III–dependent transcription require the SNAPc (*33*). We found that PK3 likely phosphorylates TRF4 of the pre-initiation complex during SLS; however, in mammals, casein kinase 2 (CK2) phosphorylates SNAP190 (SNAP2 in *T. brucei*), in a serine-rich region located adjacent to its Myb DNA binding domain, and this inhibits the ability of the SNAPc to recognize the proximal promoter (*32*). TRF4 forms a stable complex with SNAPc and TFIIA, is required to form the promoter-binding complex, and may directly interact with the promoter of *SL RNA* (*7*, *8*). In mammals, TBP does not directly interact with the promoters of snRNA encoding genes that recruit RNA pol II (*33*). Phosphorylation of the N terminus of mammalian TBP stimulates (rather than inhibits) RNA pol II–dependent transcription (*34*). In contrast, DNA damage induces CK2-mediated phosphorylation of TBP on Ser^{128}, which inhibits RNA pol III–dependent transcription of snRNA *U6* (*35*). Ser^{128} of TBP is in a different region of the protein from the homologous region containing Ser^{35} of TRF4. Thus, it is unclear whether these divergent phosphorylation events on components of the transcription initiation complex in *T. brucei* and mammals have any functional redundancy.

Our data demonstrate that PK3 is essential for SLS and PCD. However, PK3 may have substrates in addition to TRF4 that induce PCD. Complete inhibition of trans-splicing in cells depleted of the small nuclear ribonucleoprotein core complex component SmD1 increases the amount of *SL RNA*, but does not induce PCD, showing that these two processes can be uncoupled (*36*, *37*). Thus, the induction of PCD by persistent ER stress may not be a direct result of the cessation of trans-splicing but rather the consequence of additional signaling events that lead to the activation of enzymes that execute PCD. Further understanding of the role of PK3 in SLS and PCD awaits the identification of PK3-associated proteins during these processes.

In summary, this study shows that the PK3 kinase transmits the ER stress signal to the nucleus, and provides strong evidence that TRF4 phosphorylation is the main target of this response, leading to disassembly of the RNA pol II transcription preinitiation complex and cessation of *SL RNA* gene transcription. In addition, because PK3 activation triggers PCD, we believe that this finding identifies a novel factor involved in *T. brucei* PCD.

MATERIALS AND METHODS

Cell growth and transfection

Procyclic *T. brucei* strain 29-13, which carries integrated genes for the T7 RNA polymerase and the tetracycline repressor, was grown and transfected as described previously (*37*). To generate the YFP-tagged constructs, polymerase chain reaction (PCR) fragments were amplified using the primers listed in table S2.

The fragments were cloned into the p2675-YFP vector as previously described (*38*). Constructs for tagging proteins with the composite PTP tag (protein C epitope, a tobacco etch virus protease recognition site, and tandem protein A domains) were generated by amplifying the gene of interest and cloning the products into the Apa I and Not I sites of pC-PTP-BLA, which is a derivative of pC-PTP-NEO (*39*), in which the neomycin phosphotransferase coding region was replaced by that of blasticidin S deaminase. Linearization sites for targeted integration of plasmids are listed in table S2. Silencing of *PK3* was achieved using the stem-loop construct (*40*), and the primers used to generate the construct are specified in table S2. *SEC63* silencing was previously described (*18*). All constructs were verified by complete sequencing of the open reading frames.

Affinity purification and MS

Cells expressing PTP-tagged proteins, uninduced or induced for 2.5 days, were grown to a density of 2×10^7 cells/ml. The purification protocol used was essentially as described in (*41*), from 10^{10} cells, but included the addition of phosphatase inhibitors to the extract (10 mM NaF, 1 mM sodium orthovanadate, and 50 mM β-glycerophosphate). After separation of purified proteins by denaturing SDS–polyacrylamide gel electrophoresis (SDS-PAGE), protein bands were eluted from the gel and subjected to trypsin digestion. The resulting peptides were resolved by reversed-phase chromatography. MS was performed by an ion-trap mass spectrometer (Orbitrap XL, Thermo). To analyze phosphopeptides, multistage activation was used. The MS data were analyzed using Proteome Discoverer software version 1.2 or 1.3 against the *T. brucei* TriTryp database version 5 (http://tritrypdb.org/tritrypdb/).

Chromatin immunoprecipitation

ChIP was carried out as described (*42*). The immunoprecipitated DNA after cross-link reversal was deproteinized and subjected to PCR analysis using the primers listed in table S2. Antibodies to green fluorescent protein were purchased from Invitrogen (cat. no. A11122).

Western blot

Whole-cell lysates (10^6 cell equivalents per lane) were fractionated by SDS-PAGE and transferred onto Protran membranes (Whatman). The antibodies used in this study were rabbit immunoglobulin G (IgG) (Bio-Rad, 170-6515), mouse IgG (Santa Cruz Biotechnology, SC-2005), EP (Cedarlane, CLP-001A), and PTB1, PTB2, and p58 (*43*). Antibodies targeting rabbit IgG were used to detect protein A in the PTP-tagged proteins.

Northern blot analysis

Total RNA was extracted and separated on agarose-formaldehyde gel or 6% polyacrylamide gel containing 7 M urea. The blots were probed with DNA probes, which were prepared by random labeling (*37*).

Immunofluorescence

Cells were washed with phosphate-buffered saline (PBS), mounted on poly-L-lysine–coated slides, and fixed in 4% formaldehyde. Immunofluorescence was performed as described by Lustig *et al.* (*17*). Cells were visualized by a Nikon eclipse 90i microscope with a Retiga 2000R (QImaging) camera. SRP19 antibodies were prepared in our laboratory, as previously described (*44*), and the BiP antibodies were provided by J. Bang [State University of New York (SUNY), Albany, NY] (*44*).

Flow cytometry for PCD

Cells were fixed in 70% ethanol–30% PBS and stored at 4°C overnight. Cells were then washed once with PBS and incubated on ice for 30 min to enable rehydration. The samples were resuspended in PBS containing ribonuclease A (50 μg/ml; Roche Diagnostics) for 30 min at 4°C and stained with PI (50 μg/ml; Sigma). Nonpermeabilized live cells were reacted with fluorescein isothiocyanate–labeled annexin V antibodies (MBL Inc.) and stained with PI according to the manufacturer's instructions. Samples were analyzed by flow cytometry with FACStar Plus (Becton Dickinson) and CellQuest list mode analysis software (*16*).

Transmission electron microscopy

Cells were fixed with Karnovsky buffer (4% paraformaldehyde, 2.5% glutaraldehyde), essentially as described (*16*). For immunogold analysis, sections were floated in blocking buffer [10 mM tris (pH 8.5), 0.05% Tween 20, 5% nonfat dry milk] for 30 min, then incubated with rabbit antibodies that were diluted (1:20) in blocking buffer (4°C, overnight), washed with wash buffer [10 mM tris (pH 8.5), 0.05% Tween 20], and incubated with donkey antibodies against rabbit IgG (H + L) conjugated to 18-nm gold particles (Jackson ImmunoResearch). Samples were visualized with a transmission electron microscope (TEM-FEI Tecnai G2, 120 kV).

In vitro kinase assays

PK3-PTP and TRF4-PTP were affinity-purified as described above. For each kinase assay, purified proteins were incubated in 20 mM tris-HCl, 30 mM KCl, 4% sucrose, 10 mM $MgCl_2$, 4 mM dithiothreitol, and bovine serum albumin (100 μg/ml). Reactions were conducted in a 20-μl volume with 5 μCi of [γ-^{32}P]adenosine triphosphate (500 Ci/mmol) and incubated for 30 min at 37°C. The reactions were separated on 10% SDS-polyacrylamide gels and subjected to autoradiography.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/7/341/ra85/DC1

Fig. S1. TRF4 has diffuse nuclear localization in cells in low pH.

Fig. S2. In vitro kinase assays for PK3 and TRF4.

Fig. S3. Protein translocation defects in cells with *SEC63* silencing are not affected by *PK3* silencing.

Table S1. MS data (provided in Excel format).

Table S2. Primers.

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