

# The canonical pathway for selenocysteine insertion is dispensable in *Trypanosomes*

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The micronutrient selenium is found in proteins as selenocysteine (Sec), the 21st amino acid cotranslationally inserted in response to a UGA codon. In vitro studies in archaea and mouse showed that Sec-tRNA<sup>Sec</sup> formation is a 3-step process starting with serylation of tRNA<sup>Sec</sup> by seryl-tRNA synthetase (SerRS), phosphorylation of serine to form phosphoserine (Sep)-tRNA<sup>Sec</sup> by phosphoseryl-tRNA<sup>Sec</sup> kinase (PSTK), and conversion to Sec-tRNA<sup>Sec</sup> by Sep-tRNA:Sec-tRNA synthase (SepSecS). However, a complete study of eukaryotic selenoprotein synthesis has been lacking. Here, we present an analysis of Sec-tRNA<sup>Sec</sup> formation in the parasitic protozoan *Trypanosoma brucei* in vivo. Null mutants of either PSTK or SepSecS abolished selenoprotein synthesis, demonstrating the essentiality of both enzymes for Sec-tRNA<sup>Sec</sup> formation. Growth of the 2 knockout strains was not impaired; thus, unlike mammals, trypanosomes do not require selenoproteins for viability. Analysis of conditional RNAi strains showed that SerRS, selenophosphate synthase, and the Sec-specific elongation factor, EFSec, are also essential for selenoprotein synthesis. These results with *T. brucei* imply that eukaryotes have a single pathway of Sec-tRNA<sup>Sec</sup> synthesis that requires Sep-tRNA<sup>Sec</sup> as an intermediate.

phosphoseryl-tRNA<sup>Sec</sup> kinase | selenocysteine tRNA |  
Sep-tRNA:Sec-tRNA synthase | *Trypanosoma brucei* | selenoprotein

Selenium is an essential dietary trace element. It is present in proteins as selenocysteine (Sec), a cotranslationally-inserted amino acid encoded by UGA. Sec is not attached directly to tRNA<sup>Sec</sup>, but is formed by the tRNA-dependent conversion of serine (reviewed in refs. 1–4). In the first step tRNA<sup>Sec</sup> is misacylated by seryl-tRNA synthetase (SerRS). The subsequent conversion to Sec proceeds by 2 different pathways in nature. In bacteria Ser-tRNA<sup>Sec</sup> is directly transformed to Sec-tRNA<sup>Sec</sup> in a pyridoxal-5'-phosphate-dependent reaction by Sec synthase, the *selA* gene product (5). Archaea and eukaryotes require an additional step, the formation of the intermediate phosphoserine (Sep) by phosphoseryl-tRNA<sup>Sec</sup> kinase (PSTK). The resultant Sep-tRNA<sup>Sec</sup> is then converted into the Sec-tRNA<sup>Sec</sup> by the pyridoxal-5'-phosphate-dependent enzyme Sep-tRNA:Sec-tRNA synthase (SepSecS) (6, 7). The Se donor for this reaction, selenophosphate, is synthesized by selenophosphate synthase (SPS2) (8). Then EFSec, the tRNA<sup>Sec</sup>-specific elongation factor, carries the Sec-tRNA<sup>Sec</sup> to the ribosome (9) where a translational recoding process allows UGA to be read as Sec (Fig. 1). Selenoproteins are found in organisms from all 3 domains of life. Humans have 25 selenoproteins, many of them are essential for organismal viability (10). Some selenoproteins are predicted to be redox proteins containing catalytic Sec residues (e.g., glutathione peroxidase or thioredoxin reductase).

Most of our knowledge of eukaryotic Sec-tRNA<sup>Sec</sup> formation comes from in vitro reconstitution experiments using components of mammalian cells (7). In vivo formation of eukaryotic Sec-tRNA<sup>Sec</sup> has been addressed in a study that showed that RNAi-mediated ablation of SepSecS in mammalian cells did not completely abolish selenoprotein expression (11). Thus, the

existence of an alternative SepSecS-independent pathway for Sec-tRNA<sup>Sec</sup> formation could not be excluded. Moreover, the in vivo role of PSTK has not yet been analyzed.

Here, we present a comprehensive analysis of the in vivo role of the 5 major proteins required for eukaryotic Sec-tRNA<sup>Sec</sup> formation and function. The study was done in the insect form of the parasitic protozoan *Trypanosoma brucei* as double allelic KO cell lines can easily be produced in this system by homologous recombination-directed gene replacements (12). Because *T. brucei* is diploid, double KO cell lines are null mutants in both alleles. Furthermore, RNAi-based methods for highly efficient inducible ablation of proteins are also available (13). Moreover, *T. brucei* is an excellent model for exploring eukaryotic diversity. It represents a different branch of the eukaryotic evolutionary tree than the phylogenetically more closely related classical model organisms (e.g., mouse, *Drosophila*, *Caenorhabditis elegans*, and yeast) (14).

## Results

***T. brucei* Components Involved in Selenoprotein Formation.** A bioinformatic analysis of the *T. brucei* genome predicts 3 selenoproteins. They include distant homologs of mammalian SelK and SelT and a selenoprotein, termed SelTryp, that is specific for the kinetoplastid line (15). Moreover, *in silico* screens by several groups have identified Tb-tRNA<sup>Sec</sup>, Tb-SerRS, Tb-PSTK, Tb-SepSecS, Tb-SPS2, and Tb-EFSec, the trypanosomal orthologues of essentially all major components of the Sec-inserting system (16). However, of these only Tb-SerRS, Tb-tRNA<sup>Sec</sup> (16–18), and Tb-SPS2 (19) have been subject to preliminary experimental analyses.

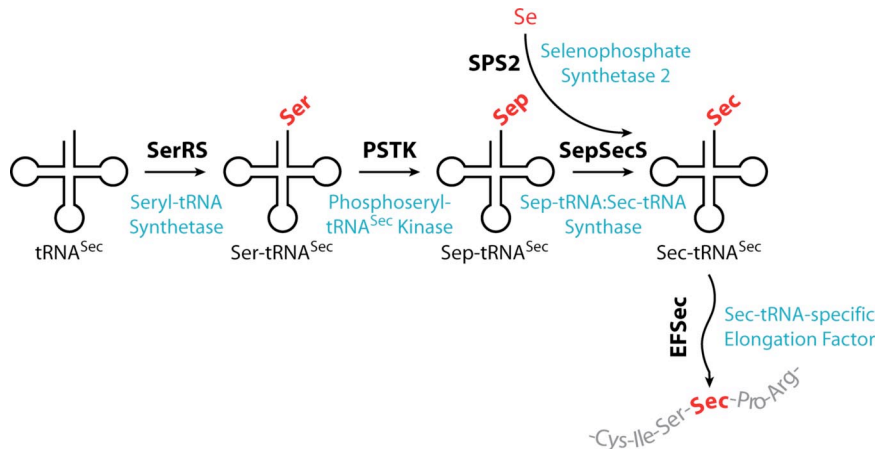
To analyze the Sec-tRNA<sup>Sec</sup> formation pathway in vivo and establish its physiological importance for *T. brucei* we used RNAi cell lines allowing inducible ablation of Tb-SerRS, Tb-SPS2, and Tb-EFSec. Moreover, we prepared KO cell lines that lack either Tb-PSTK or Tb-SepSecS, the 2 core components of the eukaryotic Sec-tRNA<sup>Sec</sup> formation pathway (Fig. S1). All cell lines were analyzed for selenoprotein synthesis by labeling with radioactive <sup>75</sup>Se (Fig. 2). The Tb-SerRS-RNAi cell line had been analyzed before by other methods, and it was shown that Tb-SerRS activity is required to charge the Tb-tRNA<sup>Sec</sup> with serine (18). Labeling of uninduced Tb-SerRS-RNAi cells with <sup>75</sup>Se and subsequent analysis by Tris-Tricine polyacrylamide gels revealed 3 bands whose molecular mass are consistent with the 3 predicted trypanosomal selenoproteins SelK, SelT, and SelTryp (Fig. 2A). SelTryp migrated in some experiments slightly faster

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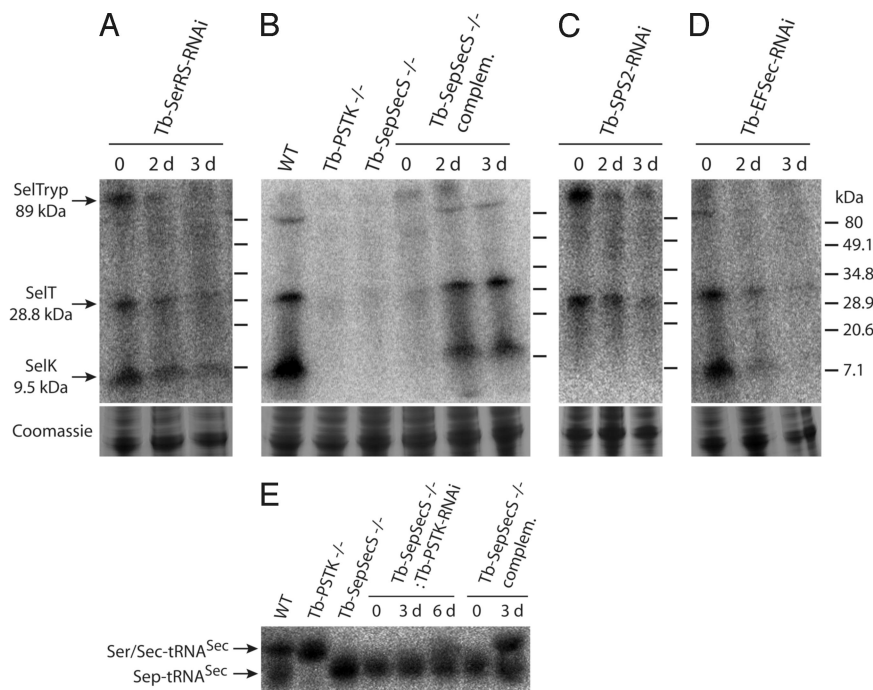
**Fig. 1.** tRNA<sup>Sec</sup>-dependent amino acid transformations leading to Sec in eukaryotes as elucidated by using recombinantly produced mammalian components (6, 7).

than expected (Fig. 2B and C). We do not know why but the simplest explanation is proteolytic clipping during sample preparation, which could also account for the simultaneous presence of both bands as seen in Fig. 2B, lane 2d.

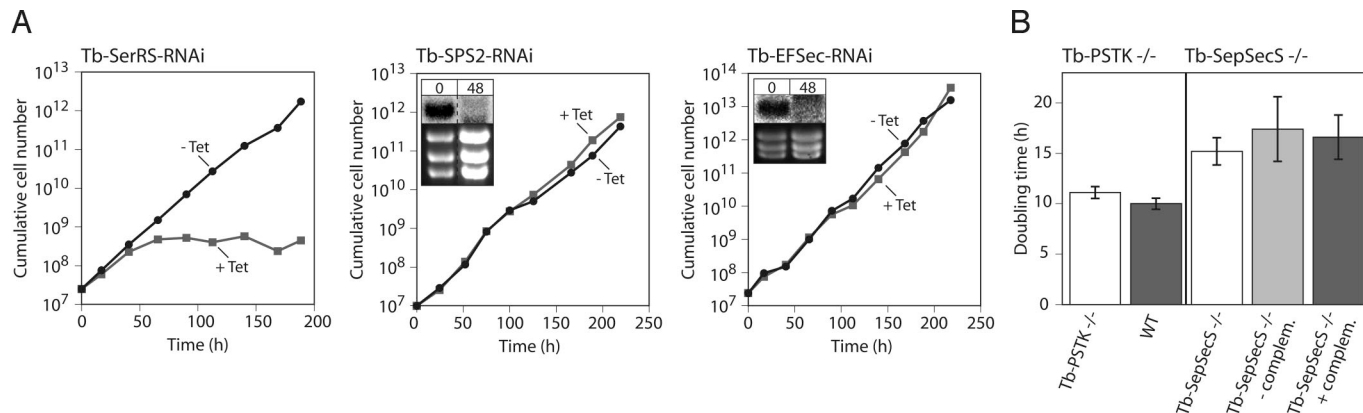
Induction of Tb-SerRS-RNAi causes, in line with the role of SerRS in serylation of tRNA<sup>Sec</sup>, an efficient but incomplete reduction of the selenoprotein labeling (Fig. 2A). This finding is in contrast to the Tb-PSTK KO and Tb-SepSecS KO cell lines in which the labeling of the 3 selenoproteins was abolished (Fig. 2B). Moreover, tetracycline-inducible ectopic expression of Tb-

SepSecS in the Tb-SepSecS KO cell line restored <sup>75</sup>Se labeling of all 3 proteins. In the case of SelTryp and SelT the restoration was to wild-type level, whereas in the case of SelK the complementation was not quantitative. These experiments show that both Tb-PSTK and Tb-SepSecS are indispensable for selenoprotein synthesis.

Next, we isolated and analyzed aminoacylated tRNAs from the different cell lines by acid urea polyacrylamide electrophoresis (Fig. 2E) (20). This technique allows the separation of the faster-migrating Sep-tRNA<sup>Sec</sup> from the slower-migrating Ser-



**Fig. 2.** Selenoprotein expression in various *T. brucei* cell lines. Expression was analyzed by <sup>75</sup>Se labeling of living cells and subsequent analysis of the labeled proteins by 10–20% polyacrylamide Tris-Tricine gels. The following cell lines were analyzed. (A) Uninduced and induced *Tb-SerRS-RNAi* cells. (B) WT 427 cells, *Tb-PSTK* KO cells (*Tb-PSTK*<sup>-/-</sup>), *Tb-SepSecS* KO cells (*Tb-SepSecS*<sup>-/-</sup>), and a *Tb-SepSecS* KO cell line allowing inducible ectopic expression of *Tb-SepSecS* (*Tb-SepSecS*<sup>-/-</sup> complem.). (C) Uninduced and induced *Tb-SPS2-RNAi* cells. (D) Uninduced and induced *Tb-EFSec-RNAi* cells. For the RNAi and the complemented *Tb-SepSecS*<sup>-/-</sup> cell lines days of induction (d) by tetracycline are indicated. The putative identity of the 3 labeled selenoproteins and their molecular mass as predicted *in silico* are indicated on the left. Molecular mass markers are indicated on the right. Segments of the tubulin region (≈50–80 kDa) of the corresponding Coomassie-stained gels are shown as loading controls. (E) Total RNA isolated for the cell lines analyzed in B and a *Tb-SepSecS* KO line capable of RNAi-mediated ablation of *Tb-PSTK* (*Tb-SepSecS*<sup>-/-</sup>:*Tb-PSTK-RNAi*) was separated on a long acidic urea gel and analyzed for the presence of the different forms of tRNA<sup>Sec</sup> by Northern analysis (18). The positions of the Sep-tRNA<sup>Sec</sup> and the comigrating Ser/Sec-tRNA<sup>Sec</sup> are indicated.



**Fig. 3.** Trypanosomal selenoproteins are not essential. (A) Representative growth curves in standard culture medium SDM-79 of uninduced and induced (–Tet, +Tet) clonal RNAi cell lines for Tb-SerRS (redrawn from ref. 18), Tb-SPS2, and Tb-EFSec. The Northern blots verifying mRNA ablation are indicated for the cell lines not showing a growth arrest. (B) (Left) Comparisons of doubling times of the Tb-PSTK KO cell line (Tb-PSTK<sup>-/-</sup>) and the parent WT strain 427 (WT). (Right) Comparisons of doubling times of the Tb-SepSecS KO cell line (Tb-SepSecS<sup>-/-</sup>) and the corresponding cell line allowing tetracycline-dependent ectopic Tb-SepSecS expression (SepSec<sup>-/-</sup> plus or minus complem.). The parent cell line for the Tb-SepSecS KO strain is *T. brucei* 29-13 that has a longer doubling time than *T. brucei* 427. Standard errors ( $n = 6$ ) are indicated.

tRNA<sup>Sec</sup> and Sec-tRNA<sup>Sec</sup> species. Distinction between the latter 2 species is not possible as they comigrate. The Northern blot analysis in Fig. 2E shows 2 tRNA<sup>Sec</sup> bands in wild-type cells. The lower one corresponds to Sep-tRNA<sup>Sec</sup> and the upper corresponds to Sec-tRNA<sup>Sec</sup> (as inferred from the pattern observed in the PSTK and SepSecS KO cell lines). In the Tb-PSTK KO cell line only the upper band is detected, which most likely represents the Ser-tRNA<sup>Sec</sup> form, because this species is expected to accumulate in the absence of PSTK activity (see Fig. 1). In the Tb-SepSecS-KO cell line, however, only the Sep-tRNA<sup>Sec</sup> band is detected. Moreover, if Tb-PSTK is depleted by inducible RNAi in the Tb-SepSecS-KO cell line, a time-dependent partial recovery of the upper band is observed, which indicates that Ser-tRNA<sup>Sec</sup> accumulates as would be expected in the absence of both PSTK and SepSecS. Moreover, the upper band, which is absent in the KO cells, reappears if the KO cell line is complemented by inducible ectopic expression of Tb-SepSecS. Thus, the accumulation of distinct intermediates of the Sec-tRNA<sup>Sec</sup> synthesis pathway in the 2 KO cell lines together with the <sup>75</sup>Se-labeling experiments show that the Tb-PSTK and Tb-SepSecS act sequentially in the indicated order (Fig. 1).

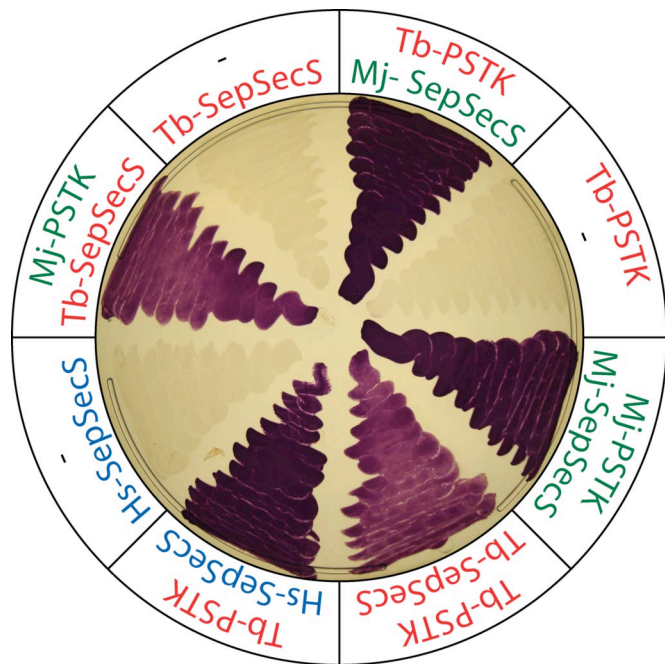
SPS2 generates selenophosphate, the Se donor required by SepSecS. Mammalian SPS2, which itself is a selenoprotein, is essential for selenoprotein synthesis *in vivo* (8). Tb-SPS2 an SPS2 orthologue capable of complementing *SelA*-deficient *Escherichia coli* has been identified in trypanosomatids (19). Like the *E. coli* orthologue, but in contrast to its mammalian counterpart, Tb-SPS2 is not a selenoprotein. Fig. 2C shows that RNAi-mediated ablation of the Tb-SPS2 severely impairs selenoprotein synthesis, indicating that SPS2 (with a cysteine in place of the Sec) is catalytically active in trypanosomes. Surprisingly, even before the induction of RNAi we reproducibly see labeling of SelTryp and SelT but not of SelK. It is possible that the SPS2-RNAi cell line is leaky and that even in the absence of tetracycline a fraction of the SPS2 mRNA is partially down-regulated. If SelK labeling is more sensitive to SPS2 levels than labeling of the other 2 trypanosomal selenoproteins its absence could be explained. Indeed selenium labeling of SelK shows a larger variation in the different cell lines than that of the other 2 selenoproteins, indicating that it might be quite sensitive to small changes of the labeling conditions (compare Fig. 2A, lane 0 with Fig. 2B, lane WT). However, without further experiments this explanation must remain speculative at present.

Sec-tRNA<sup>Sec</sup> is an elongator tRNA; however, it does not interact with elongation factor 1a but requires its own elongation factor, EFSec. Consequently, ablation of Tb-EFSec by RNAi essentially abolishes selenoprotein synthesis (Fig. 2D).

**Selenoprotein Synthesis in *T. brucei* Is Not Essential.** Selenoproteins are found in all 3 domains, indicating that they were acquired early in the evolution of life (4). However, in all domains there are many species that then lost the Sec-inserting system (e.g., fungi and plants), which raises the question of whether organisms that possess a Sec-inserting system require selenoproteins for viability. Clearly, this is the case in mammals, because a tRNA<sup>Sec</sup> KO mouse has an embryonic lethal phenotype (21). In other systems, however, this question has not been rigorously addressed. With our *T. brucei* cell lines described above we now can ask whether selenoproteins are essential for this unicellular eukaryote. Therefore, we analyzed the effects RNAi-mediated ablation of Tb-SerRS, Tb-SPS2, and Tb-EFSec (Fig. 3A) and the effects the complete lack of Tb-PSTK and Tb-SepSecS (Fig. 3B) have on *T. brucei* growth. Ablation of Tb-SerRS caused a growth arrest, which can be explained by the down-regulation of Ser-tRNA<sup>Ser</sup> levels with concomitant reduction and cessation of protein synthesis (18). Growth of all other RNAi and KO cell lines was not or only marginally impaired. For the RNAi cell lines this is not very informative because of the possible residual activity of the ablated enzymes. However, the normal growth of the Tb-PSTK and Tb-SepSecS double KO cell lines clearly demonstrates that *T. brucei* does not depend on selenoproteins.

**Conserved Sec-tRNA<sup>Sec</sup> Formation in Eukaryotes and Archaea.** When grown anaerobically *E. coli* produces selenium-dependent formate dehydrogenase (FDH). Its activity can easily be visualized on plates overlaid with benzyl viologen, because in the presence of formate active FDH reduces benzyl viologen to a blue substance (22). Using this assay it was shown that an *E. coli selA* deletion strain could be rescued by coexpression of archaeal PSTK and human or archaeal SepSecS (6). We extended these studies and showed that Tb-PSTK in combination with either endogenous *T. brucei* SepSecS or the corresponding archaeal or human enzymes reconstitute selenoprotein synthesis in an *E. coli selA* deletion strain (Fig. 4). Likewise, archaeal PSTK and Tb-SepSecS also restored FDH activity in the special *E. coli* strain. Taken together, these results underscore the highly





**Fig. 4.** Expression of SepSecS together with PSTK restores FDH activity in an anaerobically-grown *E. coli* *selA* deletion strain. The indicated genes of the following organisms were tested: *T. brucei* (Tb-PSTK, Tb-SepSecS), the Archaeon *Methanocaldococcus jannaschii* (Mj-PSTK, Mj-SepSecS), and humans (Hs-SepSecS).

conserved nature of the PSTK/SepSecS pathway of Sec-tRNA<sup>Sec</sup> formation in Archaea and eukaryotes.

## Discussion

In vitro experiments indicate that PSTK and SepSecS are the core components for Sec biosynthesis in eukaryotes (6, 7). However, the in vivo analysis of Sec-tRNA<sup>Sec</sup> formation in eukaryotes has been hampered by the lack of a suitable system. Here, we have used *T. brucei* to produce KO cell lines for the trypanosomal orthologues of PSTK and SepSecS. Analysis of these cell lines has shown that formation of Sec-tRNA<sup>Sec</sup> in living *T. brucei* requires the sequential action of Tb-PSTK and Tb-SepSecS. It remains unknown why eukaryotes use the additional phosphorylation step and thus require 2 enzymes, even though direct conversion of Ser-tRNA<sup>Sec</sup> to Sec-tRNA<sup>Sec</sup> is possible, as exemplified in bacteria (5).

Our results also show that no alternative pathway for Sec-tRNA<sup>Sec</sup> formation exists that is independent of Tb-SepSecS and Tb-PSTK. Interestingly, RNAi-mediated ablation of SepSecS had only a marginal effect on mammalian selenoprotein synthesis (11). However, the results obtained in *T. brucei* might not be readily comparable with the study in mammalian cells for the following reason. In *T. brucei* selenoprotein synthesis was assayed in a double KO cell line completely devoid of Tb-SepSecS, whereas in mammalian cells the analysis might have been obscured by the fact that RNAi cannot be relied on to completely deplete protein levels. Thus, it is likely that even in mammalian cells, just as in *T. brucei*, only a single pathway for Sec-tRNA<sup>Sec</sup> formation is operational.

Insect-stage *T. brucei* lacking selenoproteins grew as well as wild type in standard medium SDM-79, which shows that normal growth of *T. brucei* does not require selenoproteins and indicates that neither Tb-PSTK nor Tb-SepSecS have a second essential role that is unlinked to Sec-tRNA<sup>Sec</sup> formation. The unimpaired growth of selenoprotein-lacking *T. brucei* was unexpected, because it had been reported that both insect- and bloodstream-

stage *T. brucei* cells were sensitive to nanomolar concentrations of auranofin, a compound suggested to inactivate selenoproteins (15). *T. brucei* is the causative agent of human sleeping sickness. Based on the reported auranofin experiments it was proposed that selenoprotein synthesis might be a novel target for development of an antiparasitic drug (15). Because auranofin interacts with selenol and thiol groups (23), we suggest that auranofin-induced cell death might have been caused by the drug's interaction with thiols. All of our experiments were carried out in insect-stage *T. brucei* cells; therefore it is still possible that Sec is essential in *T. brucei* bloodstream forms, but it seems unlikely because all 3 predicted selenoproteins are already expressed in the insect-stage form. Thus, the prospect of using the pathway of Sec or selenoprotein synthesis as drug targets against *T. brucei* is questionable. However, because *T. brucei* in its normal habitat adapts to situations and environments that are difficult to reproduce in the laboratory, selenoproteins may play a role under such conditions.

In summary, this work illustrates that *T. brucei* is an experimentally highly-tractable system for examining the in vivo formation of eukaryotic Sec-tRNA<sup>Sec</sup>. Our analysis revealed the presence of a single pathway for Sec-tRNA<sup>Sec</sup> synthesis involving SerRS, PSTK, and SepSecS and strengthens the notion that all eukaryotes have only a single route to Sec. Furthermore, we show that selenoproteins are dispensable for insect-stage *T. brucei* under standard growth conditions.

## Materials and Methods

**Cell Culture.** Procytic *T. brucei*, strain 427 and strain 29-13, and the corresponding transgenic cell lines were grown at 27 °C in SDM-79 (24) supplemented with 5% and 15% FCS, respectively.

**Transgenic Cell Lines.** RNAi cell lines were produced by using pLew-100-based stem loop constructs containing the puromycin resistance gene (25–27). As inserts we used a 451-bp fragment (nucleotides 181–632) for the Tb-SP52 gene and a 528-bp fragment of the Tb-EF5ec gene (nucleotides 1–528). Production and initial characterization of Tb-SerRS RNAi cell line has been described (18). The double allelic replacements of the Tb-PSTK gene, in *T. brucei* 427, and of the Tb-SepSecS gene, in *T. brucei* 29–13, are described in Fig. S1. Inducible ectopic expression of Tb-SepSecS was done by using pLew-100 carrying the phleomycin resistance gene (25). Transfection, selection of transformants, and production of clonal cell lines were done by using standard procedures as described (12).

**<sup>75</sup>Selenium Labeling.** A total of  $5 \times 10^7$  cells was resuspended in 0.5 mL of FCS-supplemented SDM-79. The cultures were labeled with 9.6  $\mu$ Ci of Hepes-neutralized [<sup>75</sup>Se] selenite (University of Missouri Research Reactor, Columbia) in the presence of 100  $\mu$ g/mL cysteine at 27 °C for 3 h (for the KO strains) or 1 h (for the RNAi cell lines). After the labeling, the culture was washed with PBS, and the resulting pellet was resuspended in sample buffer and heated to 100 °C for 10 min. Finally, the labeled proteins ( $\approx 10^7$  cell equivalents) were analyzed on 10–20% Tris-tricine gels (Ready gel; Bio-Rad) and visualized with a phosphoimager.

**Acid Gel Analysis of tRNA<sup>Sec</sup> Population.** Total RNA was isolated as described (29) and resuspended in 10 mM Na-acetate, pH 4. Isolated RNA corresponding to  $8 \times 10^6$  cell equivalents was run on a 50-cm-long acidic sequencing gel as described (20). The gel was run at 4 °C in 0.1 M Na-acetate, pH 5 until the xylene blue front migrated to  $\approx 28$  cm from the top. The section of the gel containing the RNA was blotted to a Genescreen plus membrane and analyzed by Northern blots using oligonucleotide hybridization as described (28).

**Benzyl Viologen Assay for Active FDH.** Benzyl viologen-dependent FDH assays were performed as described (6). The PSTK and SepSecS genes of the different species were cloned into the pACYCDuet-1 and pET15b expression vectors (Novagen), respectively. In strains expressing only 1 gene, the corresponding empty plasmid was cotransformed as a control.

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