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Elongation factor 1a mediates the specificity of mitochondrial tRNA import in *T. brucei*

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Mitochondrial tRNA import is widespread in eukaryotes. Yet, the mechanism that determines its specificity is unknown. Previous *in vivo* experiments using the tRNAs^{Met}, tRNA^{Ile} and tRNA^{Lys} have suggested that the T-stem nucleotide pair 51:63 is the main localization determinant of tRNAs in *Trypanosoma brucei*. In the cytosol-specific initiator tRNA^{Met}, this nucleotide pair is identical to the main antideterminant that prevents interaction with cytosolic elongation factor (eEF1a). Here we show that ablation of cytosolic eEF1a, but not of initiation factor 2, inhibits mitochondrial import of newly synthesized tRNAs well before translation or growth is affected. tRNA^{Sec} is the only other cytosol-specific tRNA in *T. brucei*. It has its own elongation factor and does not bind eEF1a. However, a mutant of the tRNA^{Sec} expected to bind to eEF1a is imported into mitochondria. This import requires eEF1a and aminoacylation of the tRNA. Thus, for a tRNA to be imported into the mitochondrion of *T. brucei*, it needs to bind eEF1a, and it is this interaction that mediates the import specificity.

Keywords: elongation factor1a; mitochondrial biogenesis; selenocysteine tRNA; tRNA; trypanosomes

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Introduction

Most protozoa, many fungi, plants and a few animals lack a variable number of mitochondrial tRNA genes. It has been shown in these organisms that the missing genes are compensated for by import of a small fraction of the corresponding cytosolic tRNAs (Schneider and Marechal-Drouard, 2000; Bhattacharyya and Adhya, 2004). The phylogenetic distribution of mitochondrial tRNA import is disperse. Thus, for some species where tRNA import has been predicted, closely related organisms can be found that do not import tRNAs (Schneider and Marechal-Drouard, 2000). Since the loss of mitochondrial tRNA genes is likely to be irreversible, this suggests that the process has a polyphyletic origin. This conclusion is supported by studies of mitochondrial tRNA import in yeast (Tarassov *et al*, 1995), *Leishmania* (Goswami *et al*, 2006) and plants (Salinas *et al*, 2006), which provided evidence for three distinct tRNA import machineries. The capability to import tRNAs in these three groups of organisms is therefore due to convergent evolution.

Consistent with this view is the fact that the number of imported tRNAs is species-specific. Mitochondria of *Saccharomyces cerevisiae* import two tRNAs only (Tarassov and Martin, 1996; Rinehart *et al*, 2005). Plants import a variable number of mitochondrial tRNAs, but have retained at least a few mitochondrial tRNA genes (Dietrich *et al*, 1996b). The most extreme cases are two groups of unrelated parasitic protozoa, the trypanosomatids (which include *Trypanosoma brucei* and *Leishmania* spp.) (Simpson *et al*, 1989; Hancock and Hajduk, 1990; Schneider *et al*, 1994) and the apicomplexans (Crausaz-Esseiva *et al*, 2004b), both of which completely lack mitochondrial tRNA genes and therefore must import the whole set of tRNAs. However, in both parasites we still find tRNAs that are cytosol-specific (Crausaz-Esseiva *et al*, 2004a, b; Geslain *et al*, 2006). Interestingly, in all organisms that have been analyzed, an imported nucleus-encoded mitochondrial tRNA only represents a small fraction of a normal cytosolic tRNA (Schneider and Marechal-Drouard, 2000; Tan *et al*, 2002b). Strikingly, the imported fraction is specific for a given tRNA species and varies between 1 and 8%.

Thus, two prominent questions regarding mitochondrial targeting of tRNAs are (i) what determines the import specificity and (ii) what regulates the extent of tRNA import? Regarding the latter, it has been suggested that for some leishmanial tRNAs the extent of import is regulated by cytosol-specific thio-modifications in the anticodon (Kaneko *et al*, 2003). Regarding the former, there are a number of studies in different organisms showing that the import specificity is controlled by localization determinants on mature tRNAs (Rusconi and Cech, 1996; Entelis *et al*, 1998; Crausaz-Esseiva *et al*, 2004a). However, as expected due to the polyphyletic origin of tRNA import, they are not identical in the different species. In the imported tRNA^{Lys} isoacceptor of yeast, the localization signals are confined to the acceptor stem and the anticodon loop, and are required for binding to the precursor of mitochondrial lysyl-tRNA synthetase (Entelis *et al*, 1998). This protein forms a complex with the imported tRNA^{Lys}, which then is transported across the mitochondrial membranes by using the protein import pores (Tarassov *et al*, 1995). It is not known how the other imported yeast tRNA, the tRNA^{Gln}, is addressed to mitochondria, and by which mechanism it is imported (Rinehart *et al*, 2005).

The only other species where the *in vivo* tRNA import determinants have been analyzed in detail are *Tetrahymena* and *T. brucei*. For tRNA^{Gln} isoacceptors of *Tetrahymena* it is the anticodon (Rusconi and Cech, 1996), and for the tRNA^{Met} isoacceptors of *T. brucei* a single T-stem nucleotide pair that

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are both necessary and sufficient to determine the localization of these tRNAs (Crausaz-Esseiva *et al*, 2004a). Only fragmentary results are available for what determines the *in vivo* import specificity in plants; a point mutation in the acceptor stem of $tRNA^{Ala}$ of potato was shown to abolish import *in vivo* (Dietrich *et al*, 1996a), and more recently the D-loop and the anticodon region were implicated in import of plant $tRNA^{Val}$ (Delage *et al*, 2003).

However, it is not known in any system which factors decode the localization signals. Here we present evidence that in *T. brucei*, binding to translation elongation factor 1a (eEF1a) is a prerequiste for import, suggesting that it is this interaction that determines the specificity of tRNA import *in vivo*.

Results

Correlation between import and binding to EF1a

In *T. brucei* the initiator $tRNA^{Met}$ (Crausaz-Esseiva *et al*, 2004a) and the $tRNA^{Sec}$ are cytosol-specific (Geslain *et al*, 2006). All other tRNAs function in both the cytosol and the mitochondrion (Figure 1). Thus, by expressing chimeras between the closely related cytosolic initiator and the imported elongator $tRNAs^{Met}$, we showed that the single unmodified T-stem nucleotide pair at position 51:63 is both necessary and sufficient for the correct localization of the

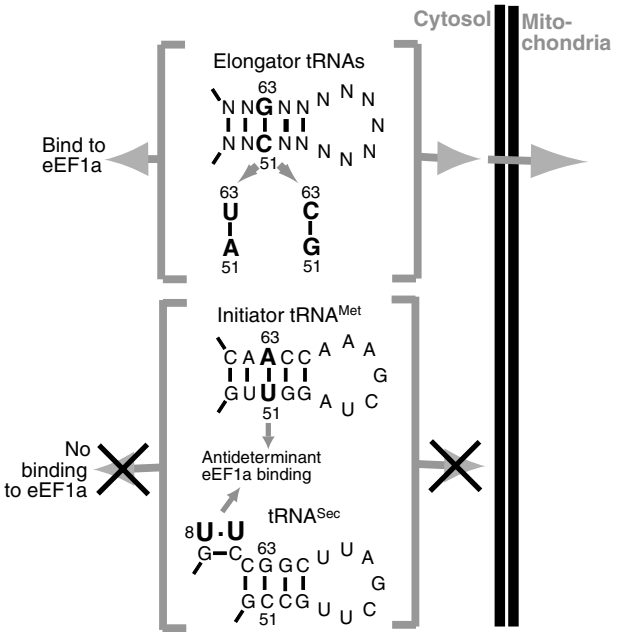

Figure 1 Specificity of mitochondrial tRNA import in *T. brucei*. Top part: All elongator tRNAs specifying the 20 standard amino acids are in part imported into mitochondria. The signal that determines their localization is the T-stem nucleotide pair C51:63G, A51:U63 or G51:C63. Lower part: The initiator $tRNA^{Met}$ and the $tRNA^{Sec}$ are cytosol-specific. The cytosolic localization signal of initiator $tRNA^{Met}$, the nucleotide pair U51:A63 (Crausaz-Esseiva *et al*, 2004a), is at the same time the major antideterminant for eEF1a binding (Drabkin *et al*, 1998). The T-stem loop region of the cytosolic $tRNA^{Sec}$ includes a putative C51:G63 import signal. However, the non-standard U:U nucleotide pair (number 8 in the acceptor stem) is a putative antideterminant for eEF1a binding (Rudinger *et al*, 1996). Thus, all tRNAs that interact with eEF1a are imported, whereas the ones that do not are cytosol-specific.

$tRNAs^{Met}$ (Crausaz-Esseiva *et al*, 2004a). The adjacent nucleotide pair 52:62 influences the efficiency of import, but when transplanted onto other tRNAs, was not able to change their localization. Furthermore, we showed that both the cytosolic as well as the mitochondrial localization determinants can act in the context of the $tRNA^{Ile}$ and the $tRNA^{Lys}$ (Crausaz-Esseiva *et al*, 2004a), suggesting that the same determinants can function in the context of any trypanosomal tRNA. Thus, if we find the T-stem nucleotide pair U51:A63, the tRNA remains in the cytosol, whereas if any other standard base pair, such as C:G, A:U or G:C, is present at this position, the tRNA is in part imported into mitochondria (Crausaz-Esseiva *et al*, 2004a) (Figure 1). (However, the $tRNA^{Sec}$ is an exception, despite carrying C51:G63 it is cytosol-specific.) Interestingly, the nucleotide pair U51:A63 is conserved in all eukaryotic initiator $tRNAs^{Met}$ and generally absent from elongator tRNAs. It not only acts as a cytosolic localization determinant in *T. brucei*, but the corresponding nucleotide pair in vertebrate initiator $tRNA^{Met}$ is one of two antideterminants that prevent binding of cytosolic eEF1a (Drabkin *et al*, 1998). The trypanosomal eEF1a is 78% identical to its human counterpart (Kaur and Ruben, 1994), which makes it very likely that the U51:A63 nucleotide pair also acts as antideterminant for the *T. brucei* protein. Furthermore, it has been shown that one tRNA domain recognized by eEF1a is the T-arm (Dreher *et al*, 1999). Thus, we observe a perfect correlation between mitochondrial import of a given trypanosomal tRNA and its predicted binding to eEF1a. This is not only true for wild-type tRNAs but also for the numerous variants whose localization we have tested *in vivo* (Crausaz-Esseiva *et al*, 2004a). In agreement with this correlation we see a congruence of the localization determinant with a nucleotide pair involved in binding or preventing of binding to eEF1a. Based on these observations we suggest the hypothesis that in *T. brucei* interaction with eEF1a is a prerequisite for a tRNA to be imported into mitochondria, and that it is this binding that determines the specificity of the process.

How does the cytosolic localization of the $tRNA^{Sec}$, which lacks the U51:A63 cytosolic localization determinant of the initiator $tRNA^{Met}$, fit into this picture (Figure 1)? It is known that $tRNAs^{Sec}$ do not bind to eEF1a (or the bacterial homologue EF-Tu). In eukaryotes this is most likely due to the non-conventional U:U nucleotide pair at position 9 of the acceptor stem, which acts as an antideterminant for eEF1a binding (Rudinger *et al*, 1996). $tRNAs^{Sec}$, instead of eEF1a, interact with their own specialized elongation factor, termed EFSec (Diamond, 2004), an orthologue of which has also been identified in *T. brucei* (Cassago *et al*, 2006; Lobanov *et al*, 2006). Taking all this into account, the cytosolic localization of the $tRNA^{Sec}$, rather than contradicting our hypothesis, actually supports it.

Inducible tRNA expression

In order to test the hypothesis that eEF1a is involved in tRNA import, we constructed RNAi cell lines allowing inducible ablation of either eEF1a or as a control of cytosolic translation initiation factor 2 (eIF2). Ablation of both of these proteins, as expected due to their essential function in translation, leads to a growth arrest but did not change the steady-state levels of mitochondrial tRNAs (data not shown). This could however be due to the fact that even in the absence of import

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the tRNA population that was imported before the induction of RNAi may persist for a long time. It might therefore not be possible to detect an import phenotype by simply analyzing the steady-state population of tRNAs. The very same problem was encountered in the analysis of mitochondrial protein import in yeast, where inducible ablation of a key import factor did not result in an obvious depletion of mitochondrial-localized proteins at steady state (Baker *et al*, 1990). However, the import phenotype was clearly seen in a pulse–chase experiment, which allows to selectively monitor newly synthesized proteins. Thus, in order to follow the fate of a newly synthesized tRNA in *T. brucei*, we produced a cell line that allows inducible expression of a nucleus-encoded and imported tRNA. Practically this was achieved by transfection of *T. brucei* 29-13, which expresses the tetracycline repressor, with a construct containing the tetracycline operator 5′ of a tagged tRNA gene. Figure 2A shows that in these cells addition of tetracycline induces expression of the tagged tRNA in a time-dependent manner. The transgenic tRNA is correctly processed as well as aminoacylated (not shown), and by all means behaves like a fully functional tRNA. Analysis of digitonin-extracted mitochondrial fractions furthermore showed that, as expected, the tagged tRNA was imported into mitochondria. *In vitro* experiments from different laboratories suggested that tRNA import requires an electrochemical gradient across the mitochondrial inner membrane (Mukherjee *et al*, 1999; Yermovsky-Kammerer and Hajduk, 1999). The left and the middle panels of Figure 2B show that treatment of a culture of *T. brucei* with carbonyl cyanide m-chlorophenylhydrazone (CCCP)—an uncoupler that dissipates the electrochemical gradient—inhibits import of the newly synthesized tRNA by 75%. This inhibition is not detected by looking at the steady-state mitochondrial tRNA pool (Figure 2B, left panel), since the major fraction of each tRNA was imported before the CCCP treatment. Staining of cells with Mitotracker (Figure 2B, right panel), a dye that detects the electrochemical gradient, confirms that incubation with CCCP depolarizes the mitochondrial inner membrane and shows that the cells remain alive and morphologically unchanged during the treatment.

Thus, these results demonstrate that *in vivo* import of trypanosomal tRNAs requires an electrochemical gradient across the inner mitochondrial membrane, and provide a proof of principle that inducible tRNA expression can be used to study aspects of mitochondrial tRNA import that previously were not accessible to direct *in vivo* analysis.

Inducible tRNA expression combined with RNAi

In a next step we produced two RNAi cell lines, which upon addition of tetracycline, induce the expression of the tagged tRNA gene, and at the same time downregulate the expression of eIF2 or eEF1a, respectively. Both cell lines showed a slow growth phenotype approximately 48 h after induction of RNAi (Figure 3). Furthermore, in both cases, concomitant with the growth arrest a reduction of cytosolic protein

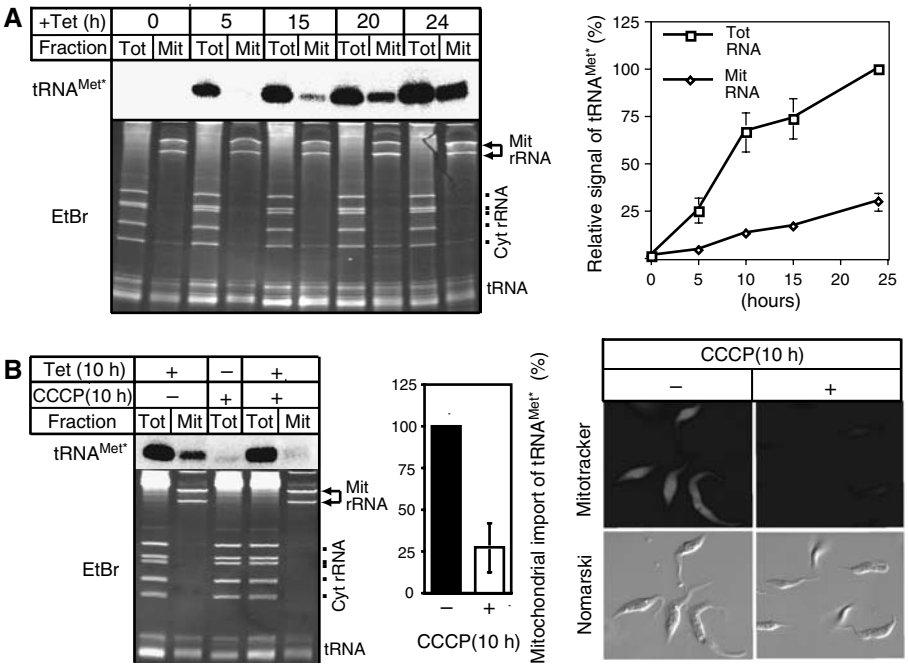

Figure 2 Tetracycline-inducible expression of a tagged tRNA. (**A**) Time course of induction. Appearance of the tagged $tRNA^{Met}$ ($tRNA^{Met*}$) in the cytosol (Tot) and in digitonin-extracted mitochondria (Mit) was monitored by Northern analysis (left side, upper panel). The lower panel shows the corresponding ethidium bromide-stained gel (EtBr). Positions of the mitochondrial rRNAs (Mit rRNA) and the cytosolic rRNAs (Cyt rRNA), as well as the tRNA region are indicated. Graph: Quantitative analysis of four independent experiments of the type shown on the left. The signal corresponding to the tagged $tRNA^{Met}$ at 24 h of induction in the total RNA fraction was set to 100%. Standard errors are indicated. (**B**) Mitochondrial import of newly synthesized tagged $tRNA^{Met}$ requires the membrane potential. Left panel: Expression of the tagged tRNA was induced for 10 h in absence (−) and presence (+) of 20 mmol of the uncoupler CCCP, and analyzed by Northern blot. Middle panel: Quantitative analysis of four independent experiments of the type shown on the left. The signal in untreated cells that corresponds to the mitochondrially localized tagged $tRNA^{Met}$ after 10 h of induction was set to 100%. Standard errors are indicated. Right panel: Mitotracker-staining of untreated (−) and CCCP-treated cells (+). The *y*-axis images of the ethidium bromide-stained gels have been electronically compressed by a factor of approximately 2.

synthesis as measured by ^{35}S-methionine incorporation was seen (Figure 3).

However, fractionation of the eEF1a-ablated cell line showed that reproducibly approximately fourfold less of the newly synthesized tRNA was found in the mitochondrial fraction than in uninduced cells (Figure 4A). In contrast, no significant effect on import of the newly synthesized tRNA was detected in cells ablated for eIF2 (Figure 4B). Most importantly, the tRNA import phenotype in the eEF1a cell line is already detected 24 h after induction of RNAi, well before growth or translation is affected (Figure 3). Consistent with this observation, the induced cells are fully motile and the electrochemical gradient of their mitochondria, as evidenced by Mitotracker staining, is identical to the one observed in uninduced cells (data not shown). As a further control, that it is indeed the lack of eEF1a that causes the

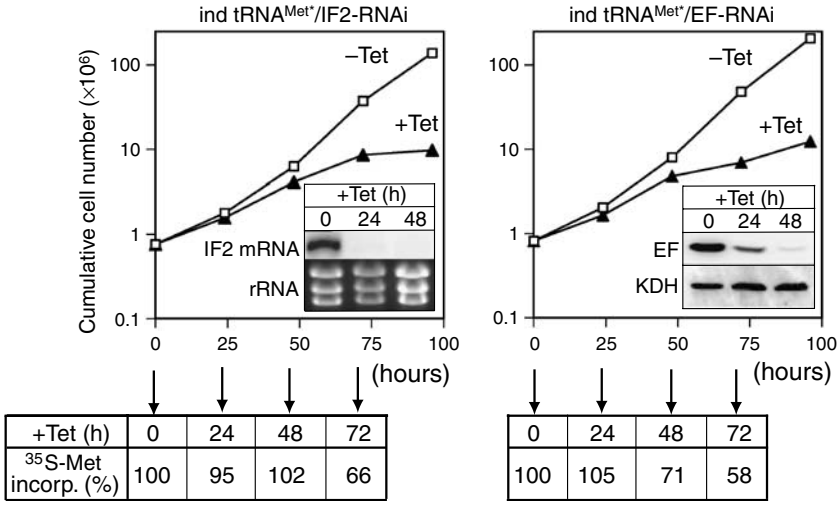

Figure 3 Inducible tRNA expression combined with RNAi. Growth curve of a representative clonal *T. brucei* RNAi cell line allowing simultaneously inducible expression of the tagged tRNA^{Met} and ablation of IF2 (ind tRNA^{Met*}/IF2-RNAi) and eEF1a (ind tRNA^{Met*}/EF-RNAi), respectively. Open squares and filled triangles represent growth in the absence or presence of tetracycline, respectively. The inset in the left graph shows a Northern blot for the eIF2 mRNA (IF2). The rRNAs in the lower panel serve as loading controls. The inset in the right panel shows an immunoblot probed for eEF1a (EF), and as control for α-ketoglutarate dehydrogenase (KDH), which is not affected by the RNAi. Quantitation of the signals illustrates that the RNAi causes efficient ablation of eEF1a relative to KDH, reaching 31 and 5% after 24 and 48 h, respectively. The efficiency of cytosolic translation during induction of RNAi expressed by the percentage of ^{35}S-labeled methionine incorporation into total cellular protein is indicated at the bottom of each graph. ^{35}S-labeled methionine incorporation in uninduced cells was set to 100%.

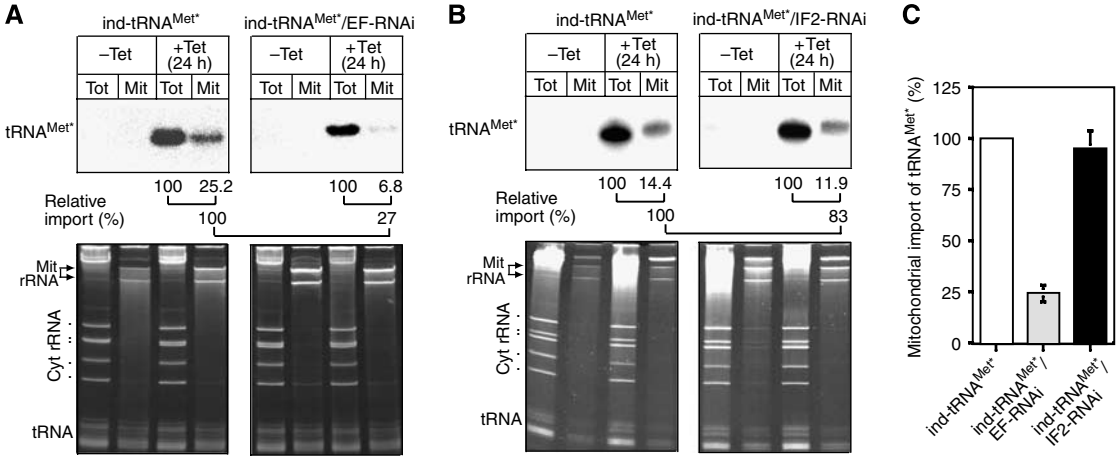

Figure 4 Effect of RNAi on import of newly synthesized tRNA. (**A**) Presence of the tagged tRNA^{Met} (tRNA^{Met*}) in the cytosol (Tot) and in digitonin-extracted mitochondria (Mit) was monitored by Northern analysis (top panels) in cell lines allowing either inducible expression of the tagged tRNA^{Met} only (ind-tRNA^{Met*}, left panel), or inducible expression of the tagged tRNA^{Met} in combination with ablation of eEF1a (ind tRNA^{Met*}/EF-RNAi, right panel). Relative import efficiency of the newly synthesized tRNA^{Met} are indicated at the bottom. The signal in the total RNA fractions (first line) or the mitochondrial fractions in the control cells (second line) was set to 100%. The lower panels show the corresponding ethidium bromide-stained gels. Positions of the mitochondrial rRNAs (Mit rRNA) and the cytosolic rRNAs (Cyt rRNA), as well as the tRNA region are indicated. (**B**) Same as panel A, but analysis on the right panel was performed with the cell lines allowing inducible expression of the tagged tRNA^{Met}, in combination with ablation of eIF2 (ind tRNA^{Met*}/IF2-RNAi, right panel). (**C**) Graph of three independent replicate experiments showing the relative import efficiencies of the newly synthesized tRNA in cells that do not undergo RNAi and in eEF1a and eIF2 ablated cell lines, respectively. Bars = s.e.

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import phenotype, we tested the import of the newly synthesized tRNA in a cell line ablated for the seryl-tRNA synthetase, an essential protein that as eIF2 and eEF1a is required for translation. It was previously shown that ablation of this enzyme causes deacylation of $tRNAs^{Ser}$ and leads to a growth arrest (Supplementary Figure 1A) whose kinetics is identical to the one seen in the eIF2 and eEF1a RNAi cell lines (Geslain *et al*, 2006) (Figure 3). According to our model we expect that ablation of the seryl-tRNA synthetase, identical to the knockdown of eIF2 but in contrast to the ablation of eEF1a, will not affect the import of the newly synthesized tRNA, which is exactly what is seen (Supplementary Figure 1B).

These results strongly suggest that inhibition of import of newly synthesized tRNAs is a direct consequence of the lack of eEF1a. However, it cannot formally be excluded that the lack of a labile factor, required for tRNA import, that is rapidly degraded under limiting eEF1a concentrations is responsible for inhibition of import.

To be imported into mitochondria tRNAs must cross both the nuclear and the mitochondrial membranes. A potential caveat of the *in vivo* import system is to distinguish nuclear retention from inhibition of mitochondrial import. There are two ways to export tRNAs from the nucleus: the exportin-t and the exportin-5 pathway (Bohnsack *et al*, 2002; Calado *et al*, 2002). There is no reason to believe that ablation of eEF1a will affect the exportin-t pathway. At first sight this looks different for the exportin-5 pathway, since it transports both tRNAs and eEF1a. However, nuclear export of eEF1a requires the presence of tRNAs that bind to both eEF1a and exportin-5. Thus, while export of eEF1a depends on tRNAs, the converse is not true and tRNAs are still exported even in the absence of eEF1a (Bohnsack *et al*, 2002; Calado *et al*, 2002). Finally, we have addressed this question experimentally for the $tRNA^{Sec}$ variants that are discussed in the next section.

In summary, inhibition of tRNA import by ablation of EF1a shows that in *T. brucei* eEF1a has a dual function; besides its role in cytosolic translation, it is required for *in vivo* import of tRNAs into mitochondria and determines the specificity of the process.

The $tRNA^{Sec}$

Eukaryotic and bacterial $tRNAs^{Sec}$ do not interact with eEF1a or EF-Tu, respectively (Diamond, 2004). Instead they have their own elongation factors. The cytosolic localization of the trypanosomal $tRNA^{Sec}$ therefore supports the hypothesis that binding to eEF1a might be a prerequisite for tRNA import. For *Escherichia coli* $tRNA^{Sec}$, the antideterminants for EF-Tu binding have been mapped to the eighth, ninth and tenth base pairs of the acceptor branch (Rudinger *et al*, 1996). Interestingly, the eighth acceptor stem base pair of eukaryotic $tRNA^{Sec}$ is invariantly a non-Watson Crick U:U (Figure 5A). It has been suggested, in analogy to the situation in bacteria, that this base pair may act as an antideterminant for eEF1a binding in eukaryotes (Rudinger *et al*, 1996). Thus, we would expect that a variant of the trypanosomal $tRNA^{Sec}$, where the U:U eEF1a antideterminant had been replaced by a standard C:G base pair should bind eEF1a (Figure 5A). Our hypothesis predicts that as a consequence this variant $tRNA^{Sec}$ should be imported into mitochondria. In transgenic *T. brucei* cells that express the variant $tRNA^{Sec}$, this is indeed observed and sequences derived from the variant $tRNA^{Sec}$, contrary to the wild-type $tRNA^{Sec}$, are recovered in both the cytosol and the mitochondrial fraction (Figure 5A). However, instead of the intact molecule we reproducibly detect two distinct smaller fragments. Thus, for unknown reasons the $tRNA^{Sec}$ variant appears to be degraded when present in mitochondria.

In a next experiment we prepared a cell line allowing inducible expression of the $tRNA^{Sec}$ variant with simultaneous knockdown of eEF1a (Figure 5B). Induction of RNAi led to a similar growth phenotype than is observed in the previously described eEF1a RNAi cell line (Figure 3) (data not shown). As expected according to our model, ablation of eEF1a for 24 h abolished mitochondrial import of the variant $tRNA^{Sec}$ (Figure 5B, left panel).

In order to show that the variant $tRNA^{Sec}$ accumulates in the cytosol and not in the nucleus, we performed cell fractionations using the detergent digitonin. A quantification of the lanes in the right panel of Figure 5B shows—after normalization to equal cell equivalents—that 50% of the primarily nucleus-localized U6 RNA is recovered in the pellet.

Figure 5 Mitochondrial import of a $tRNA^{Sec}$ variants. (**A**) Predicted secondary structure of the trypanosomal $tRNA^{Sec}$. The nucleotide changes that were introduced to obtain the variant $tRNA^{Sec}$ (var1-$tRNA^{Sec}$) that lacks the predicted eEF1a antideterminant are indicated. RNA from the cytosol (Tot) and from digitonin-extracted mitochondria (Mit) of a cell line allowing tetracycline-inducible expression of the variant $tRNA^{Sec}$ (ind-var1-$tRNA^{Sec}$) was analyzed by specific oligonucleotide hybridization for the presence of the wild-type $tRNA^{Sec}$ ($tRNA^{Sec}$) (left panel) and the variant $tRNA^{Sec}$ (var1-$tRNA^{Sec}$) (middle panel). Arrows highlight the two fragments of the variant $tRNA^{Sec}$ that are reproducibly detected in the mitochondrial fraction. Right panel: Ethidium bromide staining (EtBr) of the corresponding gel. Broken lines indicate which region of the stained gel is represented in the blot. (**B**) Effect of eEF1a-RNAi on import of newly synthesized var1-$tRNA^{Sec}$. Left panel: Northern analysis for var1-$tRNA^{Sec}$ of cytosolic (Tot) and digitonin-extracted mitochondrial (Mit) RNA fractions of an uninduced (−Tet) and induced (+Tet) cell line that allows tetracycline-regulated expression of the var1-$tRNA^{Sec}$ in combination with ablation of eEF1a (var1-$tRNA^{Sec}$/EF-RNAi). The growth phenotype of this cell line was essentially identical to the one shown for the eEF1a-ablated cell line shown in Figure 3 (data not shown). Bottom panels show a reprobing of the same blot for the endogenous imported $tRNA^{Ile}$. Middle panel: EtBr staining of the corresponding gel. Broken lines indicate which region of the stained gel correspond to which blots. Right panel: Total (Tot), cytosolic (Cyt) and nuclear (Nuc) RNA fractions were analyzed for the presence of var1-$tRNA^{Sec}$, the primarily nuclearly localized U6 RNA (U6), the cytosolic initiator $tRNA^{Met}$ ($tRNA^{Met-i}$) and for tRNAs in general (tRNAs, EtBr). The percentage of the total samples that were analyzed in the different lanes is indicated at the bottom. (**C**) Predicted secondary structure of the $tRNA^{Sec}$. The discriminator nucleotide change that prevents charging by seryl-tRNA synthetase and the nucleotide changes that inactivate the predicted eEF1a antideterminant are indicated. All these changes lead to a variant $tRNA^{Sec}$ that is termed var2-$tRNA^{Sec}$. Left panel: Total RNA from cell lines allowing tetracycline-inducible expression of the var1-$tRNA^{Sec}$ (ind-var1-$tRNA^{Sec}$) and var2-$tRNA^{Sec}$ (ind-var2-$tRNA^{Sec}$), respectively, was analyzed on a long acidic gel. Aminoacylated (aa) and deacylated (da) var1-$tRNA^{Sec}$ (left lane) and var2-$tRNA^{Sec}$ (right lane) were detected by specific oligonucleotide hybridization. Middle two panels: RNA from the cytosol (Tot) and from digitonin-extracted mitochondria (Mit) of the var2-$tRNA^{Sec}$ expressing cell line was analyzed for the presence var2-$tRNA^{Sec}$. The corresponding EtBr-stained gel is also shown. Broken lines indicate which region of the stained gel corresponds to which blot. Right panel: Distribution of var2-$tRNA^{Sec}$ in total, cytosolic and nuclear RNA fractions (as in (B)).

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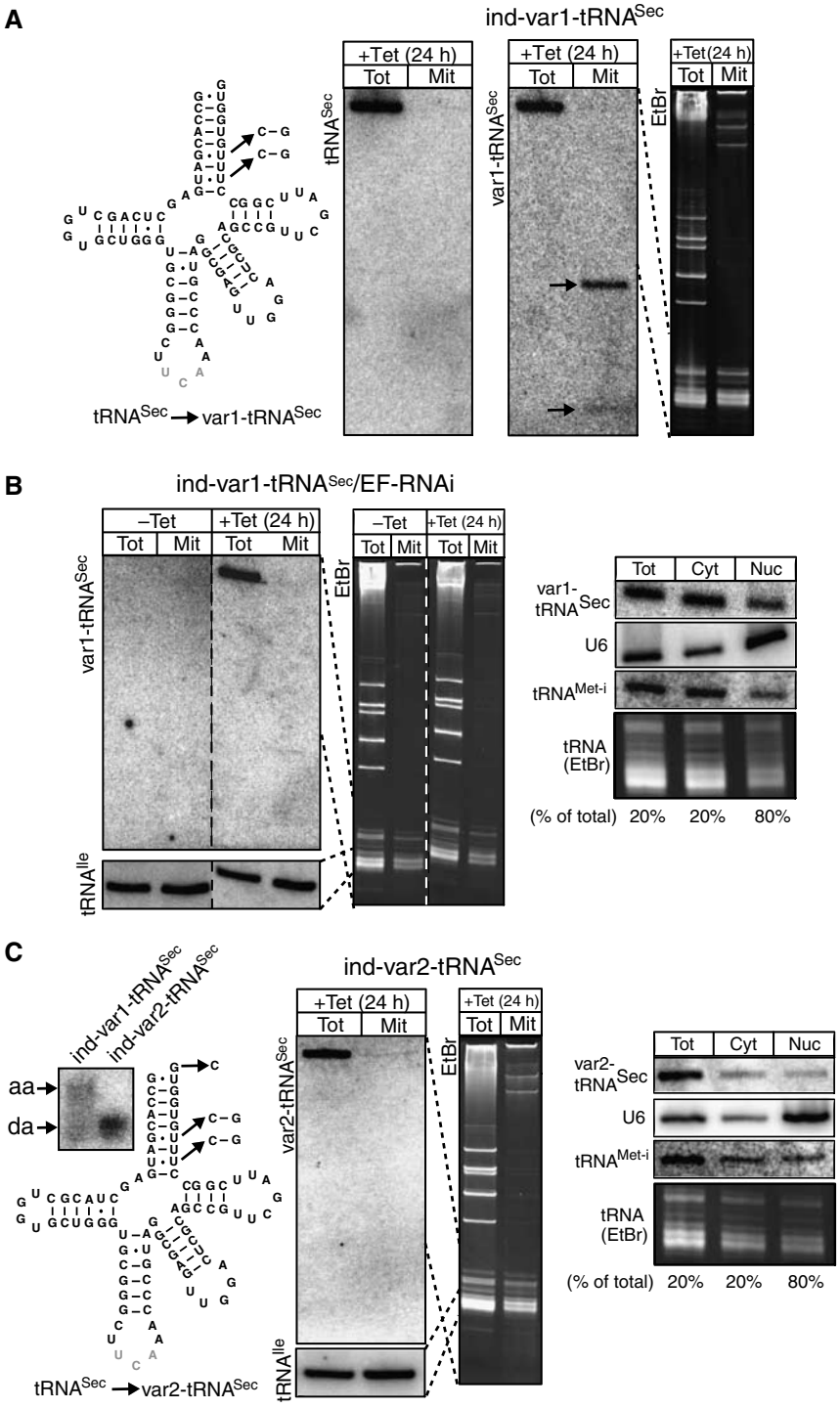
However, 88% each of the cytosolic initiator $tRNA^{Met}$ and the variant $tRNA^{Sec}$ are recovered in the supernatant, confirming that ablation of eEF1a does not interfere with nuclear tRNA export (Figure 5B, right panel).

Thus, these experiments directly link import of the variant $tRNA^{Sec}$ to the presence of eEF1a.

Formation of the ternary complex between eEF1a, GTP and tRNA requires the tRNA to be aminoacylated (Ribeiro *et al*, 1995). We have recently shown that the discriminator nucleotide G73 on $tRNA^{Ser}$ and the $tRNA^{Sec}$ is the major identity element recognized by the trypanosomal seryl-tRNA synthetase (Geslain *et al*, 2006). Thus, changing the G73 on the $tRNA^{Sec}$ to a C is expected to abolish aminoacylation. The Northern blot in the left panel of Figure 5C shows that the same is true for the $tRNA^{Sec}$ variant that is imported into mitochondria. Interestingly, cell fractionation reveals that this aminoacylation-deficient $tRNA^{Sec}$ variant cannot anymore be imported into mitochondria (Figure 5C, middle two panels) even though it lacks—just as the imported variant in Figure 5A—the antideterminant for eEF1a binding. It is important to emphasize that this experiment is not based on RNAi. Translation is therefore fully active. Quantification



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of a cell fractionation experiment (Figure 5C, right panel) shows that the absence of mitochondrial import of the variant $tRNA^{Sec}$ cannot be explained by nuclear retention of the aminoacylation-deficient $tRNA^{Sec}$. In this experiment 50% of the U6 RNA is found in the nuclear fraction, whereas 81% of the cytosolic initiator $tRNA^{Met}$ and 88% of the variant $tRNA^{Sec}$ are recovered in the cytosol.

Thus, the most parsimonious explanation for these results is that in absence of aminoacylation the tRNA cannot bind to eEF1a and therefore is not imported into mitochondria.

In summary, eEF1a-dependent mitochondrial import of the $tRNA^{Sec}$ variant that is predicted to interact with eEF1a, strongly supports the notion that this protein is required for mitochondrial tRNA import in *T. brucei*.

In vitro import shows no specificity

A number of groups have shown that efficient import of tRNAs into isolated mitochondria of trypanosomatids occurs in the absence of added cytosol and thus of eEF1a (Mukherjee *et al*, 1999; Yermovsky-Kammerer and Hajduk, 1999; Rubio *et al*, 2000; Crausaz-Esseiva *et al*, 2004a). This raises the question of how these results can be reconciled with the eEF1a-dependent targeting mechanism proposed in our model? Should the specificity of tRNA import indeed be mediated by eEF1a, we would predict that *in vitro* all tRNAs, irrespective of whether *in vivo* they are cytosol-specific or not, should be imported. In order to test this prediction, we performed *in vitro* import experiments using transcripts corresponding to the in part imported elongator $tRNA^{Met}$ as well as to the cytosol-specific initiators $tRNA^{Met}$ and the $tRNA^{Sec}$. Figure 6 shows that all these cytosol-specific tRNAs are imported into isolated mitochondria that are devoid of eEF1a. The import efficiency of the initiator $tRNA^{Met}$ is comparable to that of the elongator $tRNA^{Met}$, which *in vivo* is partly localized to the mitochondria (Crausaz-Esseiva *et al*, 2004a). Figure 6C indicates that the import efficiency does not depend on the substrate concentration. Both elongator $tRNA^{Met}$ and a variant thereof, carrying the T-stem of the *in vivo* cytosolically localized initiator $tRNA^{Met}$ (Crausaz-Esseiva *et al*, 2004a), were imported into isolated mitochondria with equal efficiency over a concentration range of 8 to 70 nM. The experimentally determined *in vivo* concentration of the initiator $tRNA^{Met}$ variant is 63 nM (Crausaz-Esseiva *et al*, 2004a), and the one of the cytosolically localized elongator $tRNA^{Met}$ variant (assayed in Figure 6C) is 520 nM, respectively (Tan *et al*, 2002b; Crausaz-Esseiva *et al*, 2004a). *In vitro* import of the *in vivo* cytosolically localized $tRNA^{Met}$ variant is therefore not due to an unphysiological high substrate concentration.

In summary these results suggest that the specificity of tRNA import is mediated by a cytosolic factor that is absent from the *in vitro* assay, and thus support our model.

Absence of eEF1a leads to unspecific import *in vitro*, but interferes with the membrane translocation step *in vivo*. This indicates that in living cells the targeting step is obligatory for the subsequent membrane translocation, whereas in the *in vitro* assay this step can be bypassed.

Discussion

The only cytosol-specific tRNAs in *T. brucei* are the initiator $tRNA^{Met}$ (Crausaz-Esseiva *et al*, 2004a) and the $tRNA^{Sec}$ (Geslain *et al*, 2006). Both of these tRNAs would be of no use inside the organelle, since the mitochondrial translation initiation mechanism is very different from the eukaryotic one (Tan *et al*, 2002a), and since no selenocysteine insertion machinery exists in mitochondria. Here we show that the lack of interaction with eEF1a provides an explanation for the cytosolic localization of these tRNAs. There are five lines of evidence supporting this conclusion: (i) an extensive *in vivo* study showed a perfect correlation of mitochondrial import of tRNAs with their predicted binding to eEF1a (Crausaz-Esseiva *et al*, 2004a); (ii) the main cytosolic localization determinant in the initiator $tRNA^{Met}$ coincides with a predicted antideterminant for eEF1a binding (Drabkin *et al*, 1998; Crausaz-Esseiva *et al*, 2004a); (iii) ablation of eEF1a abolishes import of newly synthesized tRNAs; (iv) a variant of the $tRNA^{Sec}$ that, unlike its wild-type counterpart, is predicted to bind to eEF1a, is imported into mitochondria by an eEF1a-dependent pathway (Figure 5) and (v) *in vitro* import of tRNAs in an *in vitro* system lacking eEF1a does not show specificity (Figure 6).

tRNA import can be subdivided into two temporally and spatially ordered steps. These are targeting of a subset of cytosolic tRNAs for mitochondrial import and the actual membrane translocation step. The specific interaction of eEF1a with imported tRNAs and the fact that it is a cytosolic protein that is never imported into mitochondria, indicate that eEF1a is involved in the targeting step. Thus, we suggest that besides its canonical function in translation, eEF1a selects a subpopulation of cytosolic tRNAs and hands them over to a putative receptor on the outer membrane of mitochondria. The receptor itself cannot discriminate between cytosol-specific tRNAs and tRNAs destined to be imported, which explains the lack of specificity in the *in vitro* assay (Figure 6). However, the membrane receptor appears to be able to monitor the modification status of tRNAs, as evidenced by *in vitro* import assay using *Leishmania* mitochondria. In these experiments, it was shown that the $tRNA^{Gln}$ and $tRNA^{Glu}$ carrying a thio-modified anticodon nucleotide are less efficiently imported than the corresponding tRNAs lacking it (Kaneko *et al*, 2003). Thus, while the specificity of tRNA import is controlled by cytosolic eEF1a, the extent of import might be mediated by the modification status of tRNAs.

Interestingly, unlike what one might expect, ablation of eEF1a does not selectively interfere with the targeting step, but also prevents the membrane translocation process (Figure 4). This suggests that in living cells, in contrast to the *in vitro* import assay, the targeting step is obligatory for the subsequent membrane translocation of tRNAs. In the cell line ablated for eEF1a, impairment of tRNA import occurs well before inhibition of cytosolic translation (Figure 4). This shows that determining the tRNA import specificity and translation elongation are separate functions most likely mediated by two distinct eEF1a populations. Thus, it is conceivable that a small fraction of trypanosomal eEF1a, instead of transferring the tRNA to the A-site of the ribosome, hands it over to a putative tRNA import receptor on the surface of the mitochondrion. It is at present not known whether this requires ongoing translation elongation or not. There is evidence that in eukaryotes protein synthesis is a channeled pathway, meaning that elongator aminoacyl-tRNAs are directly transferred from the aminoacyl-tRNA

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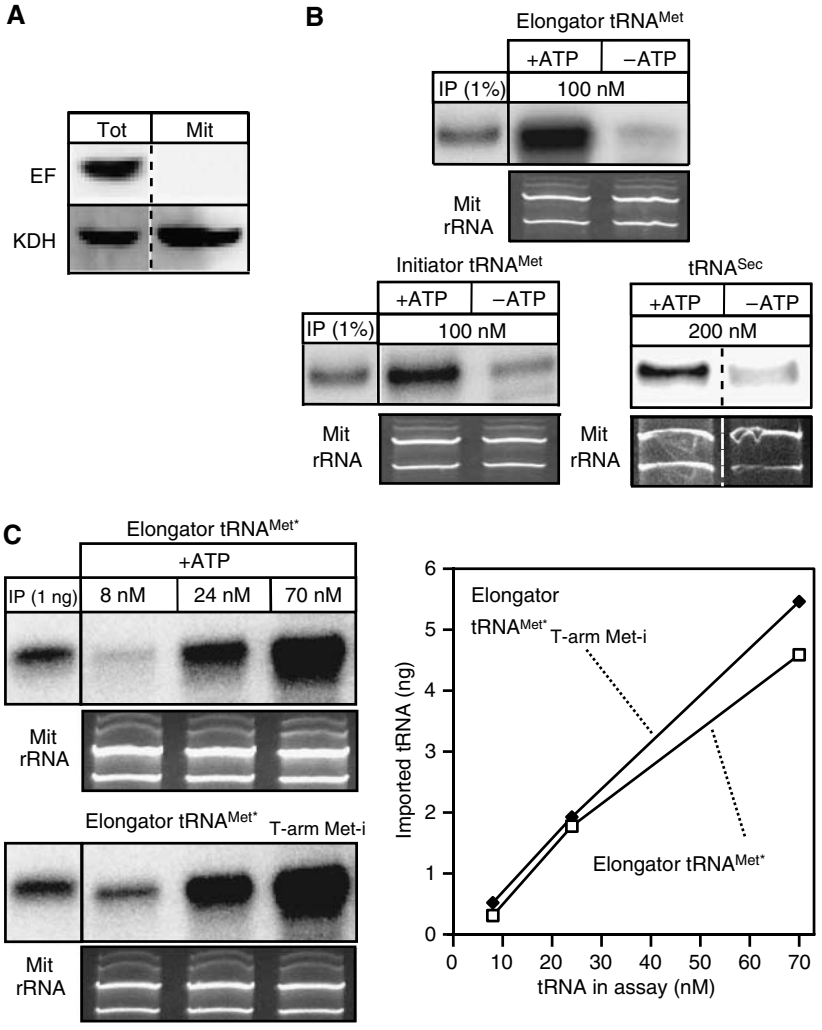

Figure 6 *In vitro* import assays. (**A**) Immunoblot analysis of 25 mg each of total cellular extract (Tot) and purified mitochondria (Mit) used in the *in vitro* assays for the presence of eEF1a (EF, top panel) and the mitochondrial marker α-ketoglutarate dehydrogenase (KDH, bottom panel), respectively (**B**) Left and middle panels: *In vitro* import in the presence and absence of ATP of *in vitro* transcribed and radioactively labeled imported elongator $tRNA^{Met}$ or cytosol-specific initiator $tRNA^{Met}$, respectively. The input lanes depict 1% of the added substrate. Right panel: *In vitro* import of *in vitro* transcribed cytosol-specific $tRNA^{Sec}$. In this case the imported $tRNA^{Sec}$ was detected by Northern blot and specific oligonucleotide hybridization. The concentration of each substrate tRNA in the import reaction is indicated. (**C**) *In vitro* import assays containing ATP and the indicated concentrations of *in vitro* transcribed tagged *in vivo* in part imported elongator $tRNA^{Met}$ (elongator $tRNA^{Met*}$, top panel) or an *in vivo* cytosol-specific variant thereof carrying the T-arm of the initiator $tRNA^{Met}$ (elongator $tRNA^{Met*}_{T-arm\ Met-i}$, bottom panel). Imported tRNAs were detected by Northern blots and hybridization of oligonucleotides directed against the tag (Crausaz-Esseiva *et al*, 2004a). The input lanes show the hybridization signals obtained by 1 ng of substrate. The graph shows the quantification of the Northern blots. All import reactions shown in panels B and C were treated with micrococcus nuclease. Ethidium bromide-stained panels show the two mitochondrial rRNAs and serve as loading controls.

synthetases to eEF1a and then to the ribosomes (Stapulionis and Deutscher, 1995; Hudder *et al*, 2003). This would mean that free aminoacyl-tRNAs that are not bound to proteins might not exist in the cell. Thus, we propose that *in vivo* import requires a highly structured cytosolic translation machinery.

However, the protein free tRNAs, that are used as substrates in the *in vitro* import system, may directly interact with the putative import receptor and as a consequence bypass the requirement for eEF1a.

Mitochondrial tRNA import is widespread among eukaryotes. However, contrary to mitochondrial protein import it has a polyphyletic evolutionary origin (Schneider and Marechal-Drouard, 2000). Thus, the tRNA import machineries might be distinct in different organisms and are probably less complex than the conserved, multicomponent protein import apparatus. This raises the question whether the recruitment of eEF1a for mitochondrial tRNA targeting is a general phenomenon? The number of tRNAs that are imported in the different systems is highly variable. In plants, for example there is no correlation of predicted eEF1a binding of tRNAs with mitochondrial import. An involvement of eEF1a in determining the tRNA import specificity is therefore unlikely. Interestingly, however, the tRNA import specificity in apicomplexan parasites, such as *Plasmodium falciparum* and *Toxoplasma gondii*, is probably identical to the one in

trypanosomatids (Crausaz-Esseiva *et al*, 2004b). Thus, the role of eEF1a in tRNA targeting might well be conserved between these two groups even though they are not obviously related.

While the targeting function of eEF1a is unlikely to be conserved in all systems that import tRNAs, it is clear that a cytosolic targeting mechanism is also operative in *S. cerevisiae*. Targeting of the imported $tRNA^{Lys}$ isoacceptor to the surface of mitochondria requires the glycolytic enzyme enolase (Entelis *et al*, 2006). Thus, comparable to eEF1a in *T. brucei*, enolase has acquired a second function. It recognizes the imported $tRNA^{Lys}$ isoacceptor and hands it over to the precursor of mitochondrial lysyl-tRNA synthetase, the carrier protein required for import.

Recruiting housekeeping components appears to be a general feature of mitochondrial tRNA import in all systems. tRNA import in yeast makes use of the protein import pathway (Tarassov and Martin, 1996). Recent studies on *Leishmania* have suggested that the alpha subunit of the F1-ATPase (Goswami *et al*, 2006) as well as subunit 6b of the ubiquinol cytochrome *c* reductase (Chatterjee *et al*, 2006) are involved in the membrane translocation of tRNAs. Finally, studies in plants have shown that the voltage-dependent anion channel of the outer mitochondrial membrane is a major component of the tRNA import machinery (Salinas *et al*, 2006). In fact up to now no factor has been described yet in any system that functions in mitochondrial tRNA import only.

A number of non-canonical functions of eEF1a have been described. It has long been known that eEF1a is an actin-binding protein. Recently, mutations in eEF1a have been produced that alter actin cytoskeleton organization without interfering with protein synthesis (Gross and Kinzy, 2005). In plants eEF1a appears to be implicated in microtubule bundling (Durso and Cyr, 1994). Furthermore, overexpression of eEF1a was proposed to be important for apoptosis (Lamberti *et al*, 2004). Our results show that in *T. brucei* eEF1a has yet another novel function, namely mediating the specificity of mitochondrial tRNA import.

Materials and methods

Inducible tRNA expression

The inducible tRNA expression system is based on the tetracycline-regulatable polymerase I promoter system originally developed by (Wirtz and Clayton, 1995). To establish the system we used a derivative of pLew-100 where the 2296-bp *Kpn*I/*Bam*HI fragment was replaced by a *Kpn*I/*Bam*HI fragment consisting of the 21-bp tetracycline operator followed by a variant initiator $tRNA^{Met}$ gene, followed by 77-bp 3′-flanking region of the wild-type initiator $tRNA^{Met}$. The variant initiator $tRNA^{Met}$ gene that was used contains the T-stem loop region of the elongator $tRNA^{Met}$ and a tag (G12:C23 to U12:A23) in the D-stem. Previous work has shown that this variant $tRNA^{Met}$ is imported into mitochondria of transgenic *T. brucei* to the same level as wild-type elongator $tRNA^{Met}$ (Crausaz-Esseiva *et al*, 2004a). Furthermore, it was shown that the tagged tRNA can be aminoacylated *in vitro* and is correctly processed *in vivo*. The construct was linearized with *Not*I and electroporated into *T. brucei* 29–13 grown in SDM-79 supplemented with 15% FCS. Transformants were selected with phleomycine and cloned as previously described (Beverley and Clayton, 1993). The selected clone allowed tetracycline-inducible (1 μg/ml) polymerase III-directed transcription of the variant initiator $tRNA^{Met}$ that could specifically be detected using the oligonucleotide 5′CGCTCTT CCCCTGAGCCA3′, which hybridizes to the region containing the D-stem tag.

RNAi cell lines

RNAi of eEF1a and eIF2 was performed using stem loop constructs containing the puromycine resistance gene, as described (Bochud-Allemann and Schneider, 2002). As inserts we used a 546-bp fragment (nucleotides 321–867) of the eEF1a gene and a 624-bp fragment of the eIF2 gene (nucleotides 257–881) (Berriman *et al*, 2005). The *Not*I-linearized constructs were electroporated into the clonal cell line obtained above. Selection with puromycine resulted in two new clonal cell lines that allow concomitant inducible expression of the variant tRNA, as well as ablation of either eEF1a or eIF2, respectively.

Expression of variant $tRNA^{Sec}$

DNA fragments consisting of the $tRNA^{Sec}$ gene, containing the changes indicated in Figure 5A or C, respectively, including 308 bp of its 5′-flanking and 205 bp of its 3′ flanking region, were cloned into a modified pLew-100 containing convenient cloning sites downstream of the procyclin promoter. Linearization, electroporation, selection with puromycine and cloning were performed as above. In the presence of 1 μg/ml tetracycline the resulting clonal cell line expresses both wild-type $tRNA^{Sec}$, which can be monitored by hybridization with the oligonucleotide 5′ACCAGCTGAGCTCAT CGTGGC3′, as well as either of the variant $tRNAs^{Sec}$, which can be specifically detected by hybridization with the oligonucleotides 5′TGGCACCACCACGGCCGA3′ (var1-$tRNA^{Sec}$) or 5′TGGGACCACC ACGGCCGA3′ (var2-$tRNA^{Sec}$).

Cell fractionation

Mitochondrial fractions were prepared by digitonin extractions (Tan *et al*, 2002b). Washed cells (4×10^8 cells each) were resuspended in 0.5 ml of SoTE (0.6 M sorbitol, 20 mM Tris–HCl, pH 7.5 and 2 mM EDTA). Five percent of the sample (25 μl) was removed to isolate the total RNA using the acidic guanidinium isothiocyanate method (Chomczyinski and Sacchi, 1987). After the addition of 0.475 ml of SoTE containing 0.1% (w/v) of digitonin, the samples were mixed by pipetting and incubated on ice for 5 min. The suspension (final concentration of digitonin 0.25%) was centrifuged (8000 *g*/5 min/4°C) and the supernatants were discarded. Next, the resulting pellets were resuspended in 500 μl of SoTE containing 1 μg of RNase A and incubated on ice for 15 min. After a final centrifugation the supernatants were discarded and RNA was isolated as for the total RNA sample. Both total RNA (corresponding to 2×10^7 cell equivalents) and mitochondrial RNA (corresponding to 2×10^7 cell equivalents) were separated on short 8 M urea/10% polyacrylamide gels.

Nuclear and cytosolic RNA fractions were prepared by digitonin extractions as described above, except that a final concentration of 0.1% of digitonin was used, 8% (w/v) of poly-(*N*-vinyl pyrrolidone) was added and no RNase digestion was performed. The nuclear RNA was isolated from the pellet and the cytosolic RNA from the supernatant. RNAs corresponding to 0.8×10^7 cell equivalents (for the cytosol) and 3.2×10^7 cell equivalents (for the nuclear fraction) were analyzed. U6 RNA was used as a nuclear marker and mature tRNAs visualized by ethidium bromide as a cytosolic marker (Figure 5B and C).

^{35}S-labeling of total cellular proteins

The two RNAi cell lines containing the inducible tRNA expression system were induced with 1 μg/ml tetracycline for the indicated time. Next the cells were washed in phosphate buffer (20 mM sodium phosphate buffer, pH 7.9, 20 mM glucose, 0.15 M NaCl) and resuspended in SDM-80 (Lamour *et al*, 2005) that lacks methionine. Subsequently ^{35}S-labeled methionine (1175 Ci/mmol) was added to a final concentration of 4 mCi/ml. After incubation for 2 h at 27°C, aliquots of 5×10^7 cells were removed and washed in phosphate buffer. The resulting pellets were resuspended in standard SDS–gel sample buffer and resolved on a 10% SDS–polyacrylamide gel. The dried gel was exposed on a phosphorimager and the total signal obtained per lane of the induced samples was compared to that of a lane containing the uninduced control.

In vitro import assays

A standard *in vitro* import reaction was performed in 20 ml of SoTE containing 2 mM DTT, 20 mM $MgCl_2$ and isotonically isolated mitochondria (500 μg protein) (Hauser *et al*, 1996; Schneider *et al*, 2007). After the addition of the indicated amounts of the different

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substrate tRNAs (Figure 6), the reaction was incubated for 20 min at 27°C in either the absence or the presence of a mixture containing 8 mM ATP, 1.3 mM creatine phosphate and 1 μg of creatine kinase (Roche). Subsequently, a tube was prepared containing a bottom layer of 10 ml of 20 mM Tris–HCl, pH 8.0, 2 mM EDTA containing 1.75 M of sucrose and a top layer of 20 μl of the same buffer containing 0.6 M of sucrose. The 20-μl import reaction was overlayed on the top layer and after centrifugation for 5 min (6800 *g* at 4°C) the top 30 μl were discarded. The remaining 20 μl were mixed by pipetting. Subsequently, $CaCl_2$ was added to a final concentration of 2.2 mM and the reaction was digested with 48 U of micrococcal nuclease (MBI Fermentas) for 15 min at 4°C, followed by 30 min at 27°C. Finally, RNA was isolated using the guanidinium isothiocyanate method (Chomczyinski and Sacchi, 1987) and analyzed on a short 8 M urea/10% polyacrylamide sequencing gel.

Miscellaneous

Transfer and Northern hybridization using specific radioactively kinased oligonucleotide probes were performed as described (Tan *et al*, 2002b).

Acknowledgements

We thank E Horn and L Bulliard for technical assistance, and also thank G Cross for providing us with cell lines and plasmids. This work was supported by grant 3100–067906 of the Swiss National Foundations and by a grant from the Velux Foundation.

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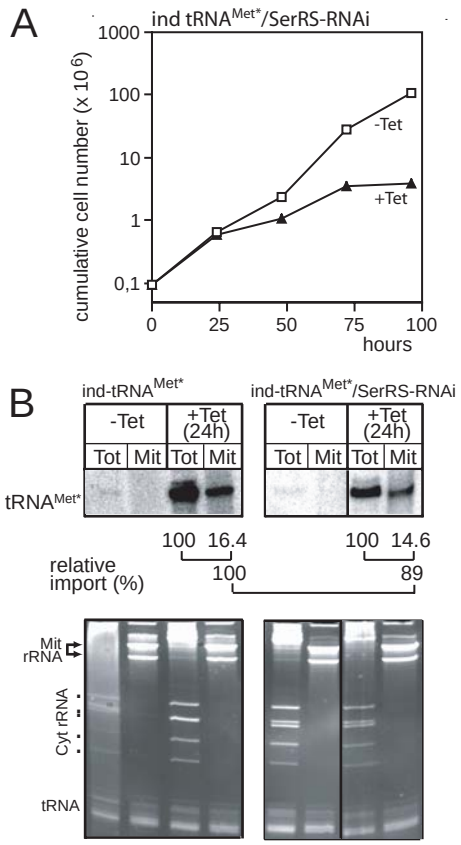

Supplementary Figure 1

Legend supplementary Fig. 1.
Inducible tRNA expression combined with ablation of seryl-tRNA synthetase. **A**. Growth curve of a representative clonal *T. brucei* RNAi cell line allowing simultaneously inducible expression of the tagged $tRNA^{Met}$ and ablation of seryl-tRNA synthetase (ind $tRNA^{Met*}$/SerRS-RNAi). The cell line is derived from the previously characterized seryl-tRNA synthetase RNAi cell line (Geslain et al., 2006). Open and filled symbols represent growth in the absence or presence of tetracycline, respectively. **B**. Effect of RNAi of ablation of seryl-tRNA synthetase on newly synthesized tRNA. Presence of the tagged $tRNA^{Met}$ ($tRNA^{Met*}$) in the cytosol (Tot) and in digitonin-extracted mitochondria (Mit) was monitored by Northern analysis (top panels) in cell lines allowing either inducible expression of the tagged $tRNA^{Met}$ only (ind-$tRNA^{Met*}$, left panel), or inducible expression of the tagged $tRNA^{Met}$ in combination with ablation of seryl-tRNA synthetase (ind $tRNA^{Met*}$/SerRS-RNAi, right panel). Relative import efficiency of the newly synthesized $tRNA^{Met}$ are indicated at the bottom. The signal in the total RNA fractions (first line) or the mitochondrial one in the control cells (second line) was set to 100%. The lower panels show the corresponding ethidiumbromide-stained gels. Positions of the mitochondrial rRNAs (Mit rRNA) and the cytosolic (Cyt rRNA) as well as the tRNA region are indicated.

Geslain, R., Aeby, E., Guitart, T., Jones, T.E., Castro de Moura, M., Charrière, F., Schneider, A. and Ribas de Pouplana, L. (2006) Trypanosoma seryl-tRNA synthetase is a metazoan-like enzyme with high affinity for tRNASec. *J. Biol. Chem.*, **281**, 38217-38225.

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