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Functions and cellular localization of cysteine desulfurase and selenocysteine lyase in *Trypanosoma brucei*

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Nfs-like proteins have cysteine desulfurase (CysD) activity, which removes sulfur (S) from cysteine, and provides S for iron–sulfur cluster assembly and the thiolation of tRNAs. These proteins also have selenocysteine lyase activity *in vitro*, and cleave selenocysteine into alanine and elemental selenium (Se). It was shown previously that the Nfs-like protein called Nfs from the parasitic protist *Trypanosoma brucei* is a genuine CysD. A second Nfs-like protein is encoded in the nuclear genome of *T. brucei*. We called this protein selenocysteine lyase (SCL) because phylogenetic analysis reveals that it is monophyletic with known eukaryotic selenocysteine lyases. The Nfs protein is located in the mitochondrion, whereas the SCL protein seems to be present in the nucleus and cytoplasm. Unexpectedly, downregulation of either Nfs or SCL protein leads to a dramatic decrease in both CysD and selenocysteine lyase activities concurrently in the mitochondrion and the cytosolic fractions. Because loss of Nfs causes a growth phenotype but loss of SCL does not, we propose that Nfs can fully complement SCL, whereas SCL can only partially replace Nfs under our growth conditions.

Structured digital abstract

- MINT-7298305: *NFS* (uniprotkb:Q386Y7) and *PHB1* (uniprotkb:Q57UX1) *colocalize* (MI:0403) by *cosedimentation through density gradients* (MI:0029)
- MINT-7298357: *SCL* (uniprotkb:Q38DC4) and *Enolase* (uniprotkb:Q38BV6) *colocalize* (MI:0403) by *cosedimentation through density gradients* (MI:0029)

Introduction

Nfs-like proteins have cysteine desulfurase (CysD) activity, and were first discovered in the nitrogen-fixing microbe *Azotobacter vinelandii*, where they are dedicated to the assembly of the iron–sulfur (Fe–S) clusters of nitrogenase [1]. These pyridoxal 5-phosphate-dependent proteins catalyze conversion of the amino acid cysteine into alanine and elemental sulfur (S) [1]. All organisms studied to date encode homologues of Nfs (termed NifS, IscS, CsdA or SufS in bacteria, depending on the gene clusters in which they are found and Nfs in mitochondria) that provide the S for Fe–S clusters. Eukaryotic Nfs proteins have a stably interacting partner Isd11, which is required for their function [2–4], and transiently interact with the scaffold protein IscU, upon which the clusters are formed [5]. Thus, the Nfs protein has a central and conserved function

Abbreviations
CysD, cysteine desulfurase; GAP1, guide RNA-binding protein 1; HA, hemagglutinin; SCL, selenocysteine lyase.

in the assembly of Fe–S clusters [6,7]. In every prokaryotic and eukaryotic cell, these ancient and omnipresent cofactors are subsequently incorporated into dozens of Fe–S proteins. These Fe–S proteins are best known for their vital role in the redox reactions during mitochondrial electron transport, but also have a similar function in photosynthesis [8], the formation of biotin and thiamine, gene expression and other cellular processes [6,7].

Moreover, many organisms contain more than one Nfs-like protein. For example, *Escherichia coli* contains three distinct Nfs-like proteins (IscS, CsdA and SufS). Although the role of CsdA in *E. coli* is not fully understood, IscS seems to have a general housekeeping role, and SufS is thought to function during oxidative stress [9]. The model plant *Arabidopsis thaliana* also encodes three functionally distinct Nfs-like proteins localized to the chloroplast, mitochondria and cytosol [10]. Two Nfs-like proteins have been identified in the apicomplexan protist *Plasmodium* [11], including one localized to the apicoplast, whereas the yeast and human genomes encode only a single Nfs-like protein. However, the human *NFS1* gene contains an alternative start site, which provides dual localization of the protein to the mitochondria or the cytosol and nucleus [12]. In similar fashion, the yeast Nfs1 protein is predominantly found in the mitochondion, but is also localized to the nucleus in small amounts, and has been shown to be indispensable for survival [13,14]. Yeast is not dependent on mitochondrial electron transport during anaerobic growth and so it is likely that the yeast Nfs1 protein is essential because of the Fe–S cluster assembly for proteins localized in the cytosol and the nucleus. Moreover, yeast Nfs1 is also necessary for the thiolation of tRNAs [15]. Indeed, mutation of the nuclear localization signal in the mature Nfs1 protein is also lethal in yeast, despite having no effect on mitochondrial Fe–S proteins. These results suggest that the yeast Nfs1 protein has an essential role in both nuclear and cytosolic Fe–S cluster assembly [15].

Interestingly, in addition to CysD activity, all Nfs-like proteins have selenocysteine lyase (SCL) activity, which cleaves selenocysteine into alanine and selenium. [16]. SCL activity is essential for organisms that require selenium, as first documented in bacteria and later in mammals, both of which contain selenoproteins [17]. Single-celled organisms, such as the green algae *Chlamydomonas reinhardtii* and *Emiliana huxleyi* are also known to contain selenoproteins [18], although their set is smaller than in mammals [19].

The genome of *Trypanosoma brucei*, the causative agent of African sleeping sickness, encodes two Nfs-like proteins [20]. Downregulation of the Nfs protein, which is confined to the mitochondrion, impaired ATP production, cellular respiration and growth, suggesting that this protein is essential for the assembly of Fe–S clusters incorporated into the mitochondrial proteins [20]. More recently, it was discovered that in trypanosomes ablated for Nfs, tRNA thiolation is disrupted [21]. Moreover, in *Saccharomyces cerevisiae* and *T. brucei*, the mitochondrially located Nfs1 and Nfs proteins, respectively, are responsible for the thiolation of tRNAs in both the mitochondria and cytoplasm [21,22]. Because *T. brucei* contains a set of selenoproteins [23–25], as well as a complete machinery for the formation of Sec–tRNA^{Sec} [26], we undertook functional characterization of cells with downregulated Nfs-like protein of the selenocysteine type.

Results

Phylogenetic analysis

A genome-wide search revealed that *T. brucei* and all other kinetoplastid flagellates, for which full genome sequences are available, contain two Nfs-like proteins in their nuclear genome. Recent evidence suggests that one of them, called Nfs (formerly TbIscS2), exhibits CysD activity and has a function in Fe–S cluster assembly similar to other well-studied homologues found in eukaryotes [20]. The second gene codes for a 451 amino acid protein with calculated molecular mass of 48.9 kDa. It contains a highly conserved PLP-binding lysine 258, the active cysteine 393 responsible for desulfuration, as well as histidine 125, which initiates the release of sulfur by deprotonation of L-cysteine. In the sequence, however, the conserved serine 255 is replaced by cysteine, and a substantial part of the active site loop, as well as the C-terminal region known to mediate interaction with IscU, are lacking. A predicted nuclear localization signal (PPLKKLR) is located in the N-terminal region of the protein sequence.

We have performed an extensive phylogenetic analysis of Nfs-like genes from *T. brucei* using maximum likelihood, maximum parsimony and neighbor joining analyses (see Experimental procedures for details). An unrooted phylogenetic tree obtained from an alignment of amino acid sequences of the Nfs/IscS and SCL genes from 90 prokaryotes and 60 eukaryotes revealed a very distant position for both *T. brucei* genes (Fig. 1). The analysis did not recover a single clade containing solely prokaryotic sequences, but rather several paraphyletic clades. Eukaryotic genes are split into two large groups of different origin,

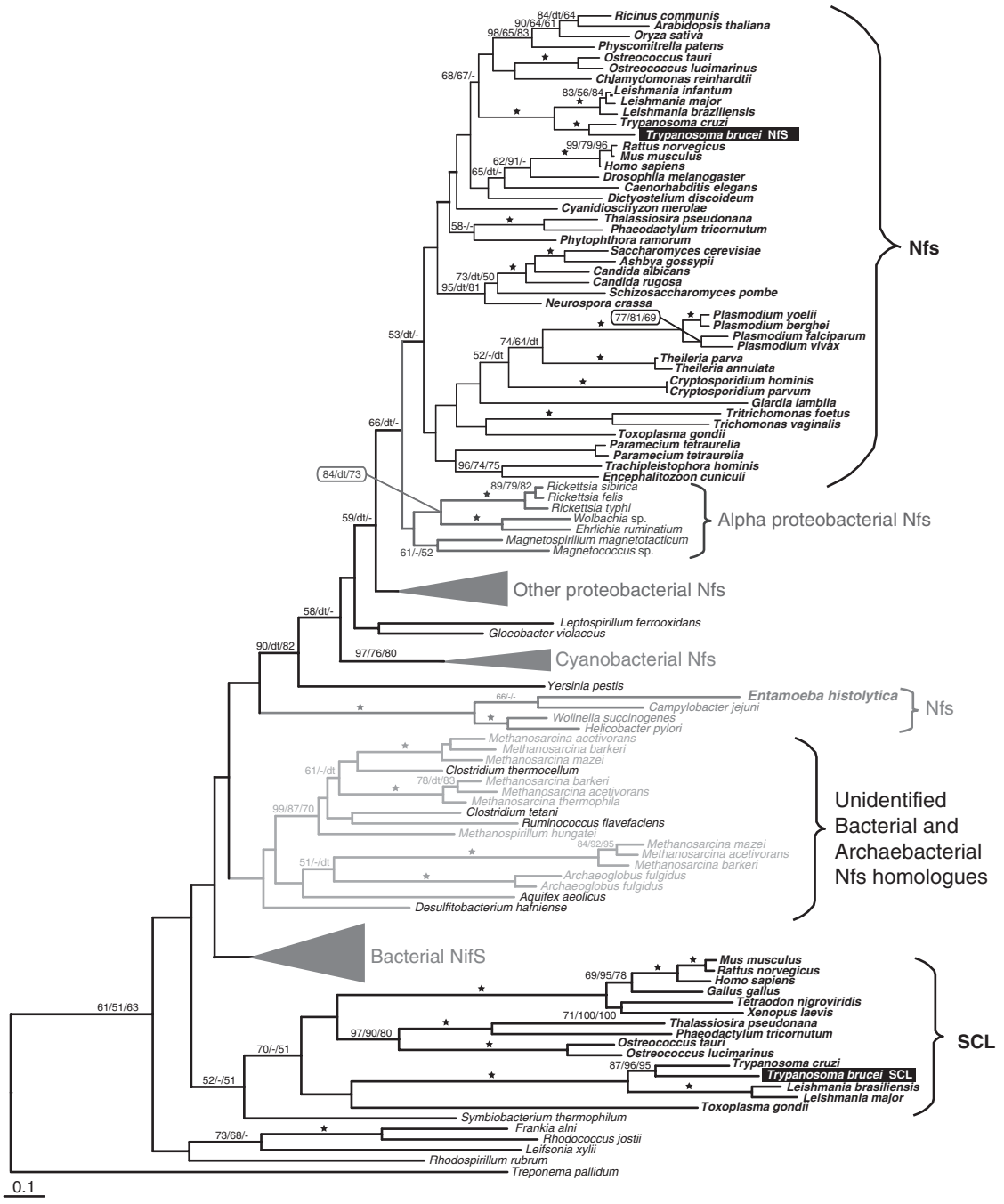

Fig. 1. Maximum likelihood phylogenetic tree as inferred from 348 amino acid positions of the Nfs/IscS and related proteins (SUFs were not included). Numbers above branches indicate maximum likelihood (ML) (300 replicates)/neighbor joining (NJ) (1000 replicates)/maximum parsimony (MP) (1000 replicates) bootstrap supports. Stars indicate branches with all bootstraps overcoming 90% 'dt', different topology for the particular method (maximum likelihood/neighbor joining/maximum parsimony). Sequences found in eukaryotes are given in bold.

interspersed with numerous prokaryotic Nfs-like sequences. The early-branching group brings together all putative eukaryotic selenocysteine lyases, which probably represents the gene originating in the eukaryotic nucleus. Consequently, this phylogenetic analysis indicates that the *T. brucei* Nfs-like gene encodes a selenocysteine lyase, and will be henceforth labeled as such (SCL).

The second well-supported group of genes contains CysDs including Nfs of *T. brucei*. (Fig. 1). However, although these Nfs genes are encoded in the eukaryotic nucleus, they likely originate from the ancestor of the mitochondrion, because α-proteobacteria constitute a robust sister group. The ancestry of the Nfs gene from the mitochondrion is thus well supported, whereas the origin of the SCL gene remains unclear. Consequently, these two genes have obviously acquired different, yet overlapping, functions in the eukaryotic cell (see below).

RNAi knockdown of SCL

An RNAi cell line was prepared by introducing into the insect (procyclic) stage of *T. brucei* strain 29-13 a pZJMβ vector containing a 415 bp fragment of the SCL gene. The criterion for the selection of this fragment was the lowest possible sequence similarity to the Nfs gene. Transfection of the procyclics resulted in stable integration and phleomycin-resistant transfectants were obtained by limiting dilution. Induction of double-stranded RNA synthesis upon the addition of tetracycline indeed resulted in efficient elimination of the SCL mRNA in two selected clones within 24 h of induction (Fig. 2A). In order to rule out the possibility that cross-reactivity also induced the downregulation of Nfs, which shares with SCL 33 and 52% identical and similar amino acids, respectively, a northern blot was performed with a probe against the Nfs gene, which confirmed that the respective mRNA is not targeted by nonspecific RNAi (Fig. 2B). Despite effective silencing, growth of the cloned procyclic cells was not inhibited upon RNAi induction with tetracycline, even when it was followed for a prolonged period of 2 weeks (Fig. 2C).

Western blot analysis with polyclonal antibodies generated against the *T. brucei* CysD Nfs and the scaffold protein IscU revealed that the ablation of the target SCL protein did not result in a detectable loss of the above-mentioned proteins even 8 days after RNAi induction (Fig. 3A). We also used anti-Nfs IgG to verify the predicted mitochondrial localization of this protein in the procyclic *T. brucei*. Indeed, the protein seems to be confined to the organelle (Fig. 3B). The purity of cellular fractions was confirmed by antibodies against cytosolic enolase and mitochondrial prohibitin (PHB1).

Localization of SCL protein

We used a tagging strategy to analyze the intracellular localization of this protein. A hemagglutinin

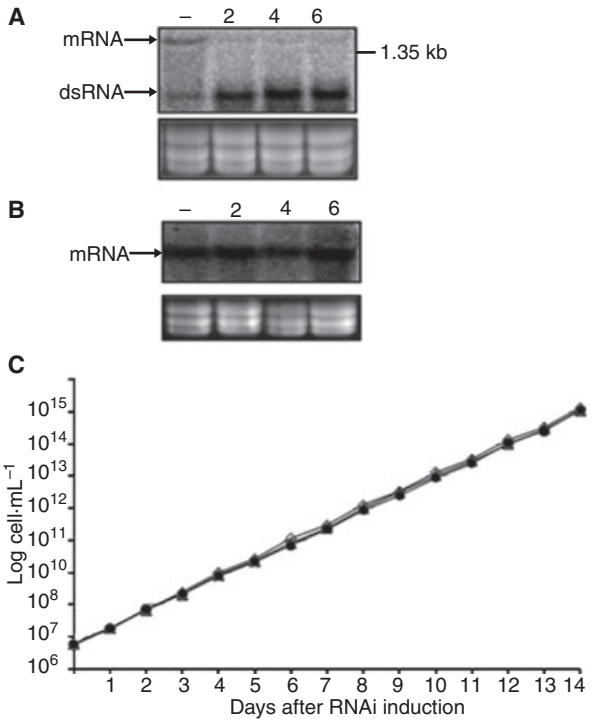

Fig. 2. Effect of SCL RNAi on mRNA levels. (A) SCL mRNA levels were analyzed by blotting total RNA extracted from the noninduced SCL cells (–) and SCL cells harvested 2, 4 and 6 days after RNAi induction. The position of the targeted mRNA and the double-stranded (ds) RNA synthesized following RNAi induction are indicated with arrows. (B) Nfs mRNA levels were analyzed in the RNA samples described in (A). As a control, both gels were stained with ethidium bromide to visualize rRNA bands. (C) Effect of SCL RNAi on cell growth, compared with 29-13 and noninduced cells. The numbers of 29-13 cells (diamonds), noninduced cells (triangles) and those induced by the addition of 1 μg·mL^{-1} tetracycline (circles) were plotted as the product of cell density and total dilution. Growth curves are one representative set from three experiments.

(HA_3) tag was attached to the C-terminus of the full-size SCL gene in a vector that allows inducible expression of the tagged protein driven by a strong procyclin promoter. The tag was placed on the C-terminus in order to not interfere with a predicted nuclear import signal usually located at the N-terminus. Subcellular fractions of the transfected procyclic cells were obtained by digitonin treatment performed, as described elsewhere [20]. As shown by western blot analysis of the total cell lysate and the mitochondrial and cytosolic fractions, tagged protein is detected only in the cytosolic fraction, which is composed of nuclei and the cytosol (Fig. 4A). Polyclonal antibodies against enolase and guide RNA-binding protein 1 (GAP1) were used as cytosolic and mitochondrial loading controls, respectively.

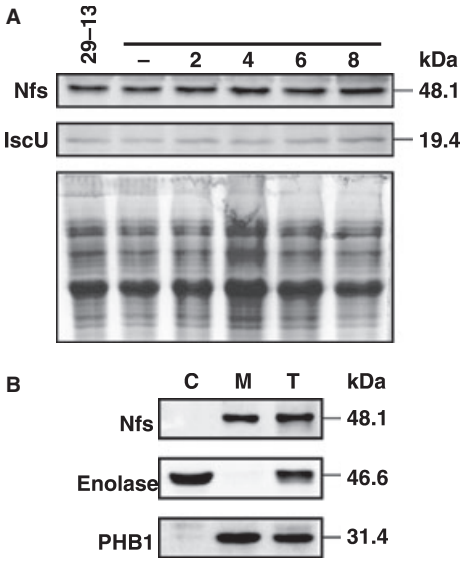

Fig. 3. Effect of SCL RNAi on protein levels and cellular localization of Nfs. (A) Nfs and IscU protein levels were analyzed by western blot analysis in extracts from 29-13 procyclics, as well as from the non-induced SCL cells (–) and SCL cells harvested 2, 4 and 6 days after induction. Coomassie Brilliant Blue staining of proteins obtained from $\sim 5 \times 10^6$ cells·lane^{-1} is shown as a loading control. (B) Nfs in localized in the mitochondrion. Western blot analysis of total (T), cytosolic (C) and mitochondrial (M) lysates immunoprobed with the polyclonal antibodies against Nfs, enolase and prohibitin (PHB1). Anti-enolase and anti-prohibitin IgG were used as cytosolic and mitochondrial markers, respectively.

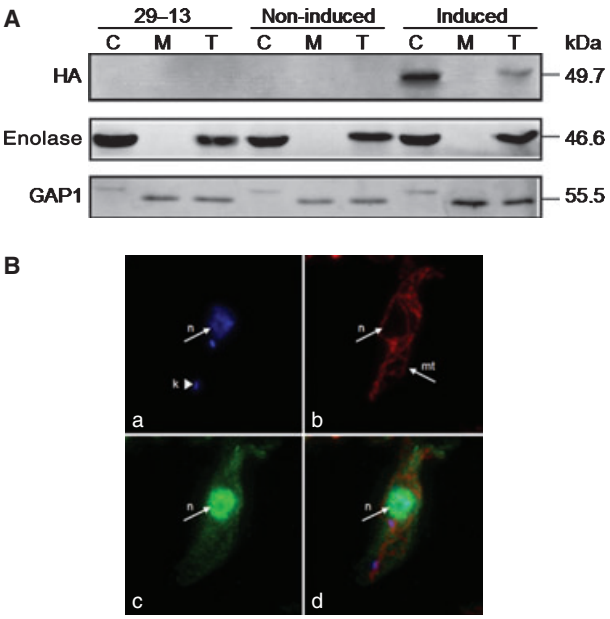

Fig. 4. Nuclear localization of inducibly expressed HA_3-tagged and TAP-tagged SCL protein, respectively. (A) Immunoblot analysis of the HA_3-tagged protein in total cell lysates (T), and cytosolic (C) and mitochondrial fractions (M) obtained from noninduced cells and cells, in which expression of HA_3-tagged SCL was induced by the addition of tetracycline. Parental 29-13 cells were used as a control. The α-GAP1 and α-enolase polyclonal antibodies were used as mitochondrial and cytosolic markers, respectively. (B) Immunolocalization of the TAP-tagged SCL protein in procyclic *T. brucei* (a) 4′,6-diamidino-2-phenylindole-staining of nuclear and kinetoplast DNA; (b) mAb mAb56 against the mitochondrial MRP1/2 complex was used to visualize the single mitochondrial network; (c) predominantly nuclear located TAP-tagged SCL protein was visualized by fluorescence microscopy using polyclonal anti-c-*myc* serum coupled with fluorescein isothiocyanate-conjugated secondary antibody; (d) merged fluorescence images. Nucleus (n) is indicated with an arrow, kinetoplast (k) with an arrowhead.

This result was further corroborated by fluorescent microscopy of tetracycline-induced cells bearing the TAP-tagged SCL gene. The cells were stained by 4′,6-diamidino-2-phenylindole and prepared for immunocytochemistry using a polyclonal α-myc antibody. Interestingly, most of the signal was observed in nuclei with some signal also distributed throughout the cytoplasm, which may imply a dual localization of the Nfs-like protein, or its presence in the cytoplasm because of its overexpression (Fig. 4B). As a control for staining of the mitochondria, the mAb mAb56 against the mitochondrial MRP1/2 complex [27] was used (Fig. 4B). MS analysis of the TAP-tagged purified SCL protein failed to identify any protein associated with it, indicating that the SCL protein has no strongly interacting partner (data not shown).

Measurement of enzymatic activities

Selenoproteins have previously been detected in the trypanosome proteome [23–25]. Because selenoprotein synthesis would require the generation of elemental Se from selenocysteine, we analyzed SCL activity in the procyclic cells. Moreover, because Nfs-like proteins can use cysteine and selenocysteine as substrate [28], we tested whether the elimination of SCL resulted in a decrease in or disruption of the SCL and CysD activities. Specific activities for the cysteine and selenocysteine substrates were measured in the noninduced and RNAi-induced knockdown cells for SCL characterized above, and also in the noninduced and Nfs RNAi-induced cells described earlier [20]. The measurements in total cell lysates showed that specific activities for both substrates are decreased in each of the knockdowns (data not shown). This experiment strongly supports the hypothesis that both proteins function as possible CysDs and may also have selenocysteine lyase activity.

To determine if the SCL and CysD activities differed in cellular compartments, cytosolic and mitochondrial protein fractions were prepared and analyzed

separately (Fig. 5). Wild-type SCL specific activity was 2.5-fold higher in the cytosol than in the single reticulated mitochondrion. Four days after RNAi induction, both cell lines with downregulated SCL or Nfs showed a decrease in the SCL specific activity in the cytosol, and to a greater extent in the mitochondrion (Fig. 5C,D). Knockdowns for Nfs, which is the procyclic *T. brucei* confined to the mitochondrion [20], had a lower SCL specific activity than cells in which SCL was ablated. Measurement of the CysD activity indicated an even more pronounced decrease. Again, in wild-type cells, this specific activity was $\sim$ 2.5-fold higher in the cytosol than in the mitochondrion. Approximately 20% and 40% of the specific activity in the cytosolic fraction was retained in the SCL and Nfs RNAi cell lines, respectively (Fig. 5A). By contrast, CysD specific activity was virtually eliminated from the mitochondrion of these cell lines, with only 11% present in the Nfs knockdowns (Fig. 5B).

In both knockdown cells, SCL and CysD activities began to increase on day 8 after RNAi induction. This general trend is expected because it is well known that *T. brucei* can become resistant to RNAi, usually after 1 week. However, it is worth noting that the SCL activity recovers more slowly in SCL than in Nfs knockdowns, and the same applies to CysD activity in the respective cells (data not shown).

Discussion

Initially, the mitochondrion was considered the sole compartment in which Fe–S clusters are generated for the entire eukaryotic cell [29]. Soon afterwards, the localization of Nfs-like proteins to the nucleus and cytosol was discovered [7,30]. Studies in plants also revealed that an independent center of Fe–S cluster synthesis is present in the chloroplast [31], which is not surprising given the evolutionary history of plant plastids and the requirement of an electron transport chain in both the mitochondrial and chloroplastic compartments. It is now becoming more apparent that the assembly of Fe–S clusters is not restricted to where the CysDs are localized. This scenario was primarily supported by the observation that the Fe–S assembly

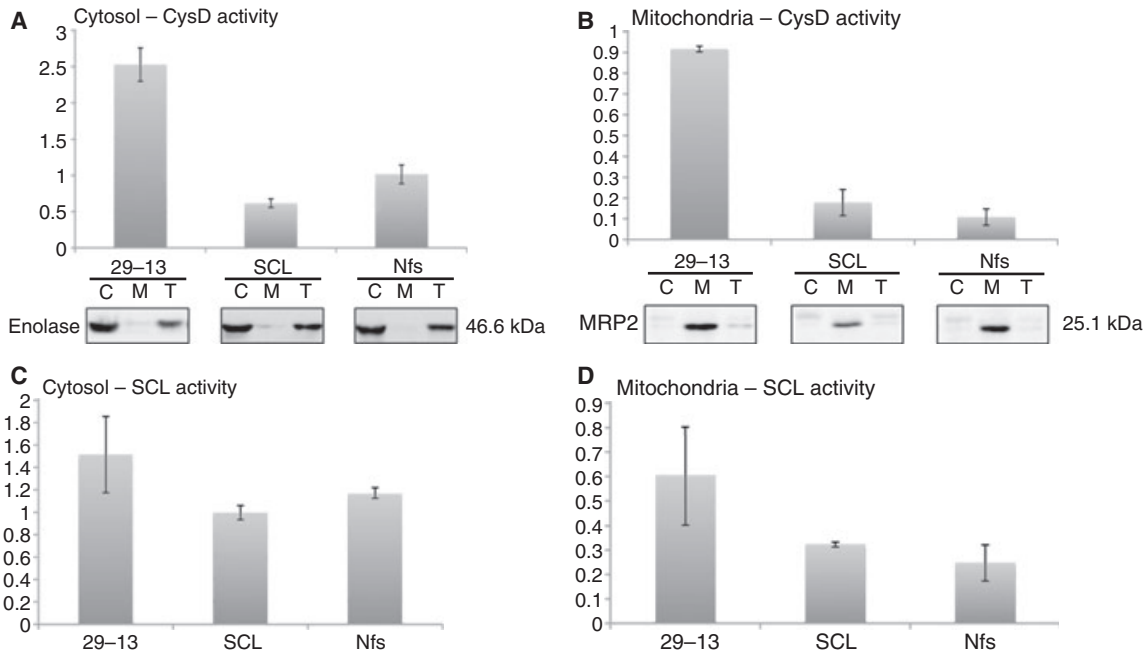

Fig. 5. Measurement of CysD and SCL specific activities. Mitochondrial and cytosolic protein extracts were obtained from parental 29-13 cells, and the Nfs and SCL knockdowns after 4 days of RNAi induction, as described in Experimental Procedures. The purity of all protein fractions used for activity measurement was controlled by western blot analysis using antibodies against mitochondrial RNA-binding protein (MRP2) and enolase, used as mitochondrial and cytosolic markers, respectively. The mean and SD values represent the averages of multiple measurements of three independent RNAi inductions. (A) Cys desulfurase activity was measured in cytosolic fractions. Units are in nmol sulfide·min^{-1}·μg protein^{-1}. (B) Cys desulfurase activity was measured in mitochondrial fractions. Units are in nmol sulfide·min^{-1}·μg protein^{-1}. (C) SCL activity was measured in cytosolic fractions. Units are in μmol selenide·min^{-1}·μg protein^{-1}. (D) SCL activity was measured in mitochondrial fractions. Units are in μmol selenide·min^{-1}·μg protein^{-1}.

in yeast appeared to depend on a mitochondrial membrane transporter [32]. An increasing amount of data now point towards the existence of a cytosolic iron–sulfur cluster assembly pathway termed CIA, which may serve the synthesis of Fe–S clusters assembled onto nuclear and cytosolic proteins [7].

As reported earlier, the downregulation of Nfs dramatically lowers the activities of mitochondrial Fe–S cluster-containing enzymes, causing a significant decrease in the growth rate of *T. brucei* procyclics [20]. Moreover, in trypanosomes, as well as in yeasts, this protein was recently shown to be indispensable for the thiolation of cytosolic and mitochondrial tRNAs [21,22,33]. Importantly, analysis of the status of tRNA thiolation in cells depleted for the SCL protein did not reveal any changes demonstrating that this enzyme is not involved in tRNA metabolism [21]. As we show in this study, after silencing of SCL, CysD activity decreases by $\sim 75\%$ in both the mitochondrion and the cytosol. Almost the same decrease is observed in cells in which Nfs was targeted by RNAi, although in the mitochondrion of these knockdowns CysD activity decreases by 90% (Fig. 5). In analogy with other eukaryotes containing selenoproteins [34], *T. brucei* was supposed to be dependent upon the SCL activity for the formation of putatively essential selenoproteins. However, recent finding suggest that selenoproteins are not needed for the survival of trypanosomes, at least under cultivation conditions [26], hinting that SCL may also be dispensable. We have confirmed this unexpected observation by experiments with auranofin, a highly specific inhibitor of selenoenzymes [23], because the downregulation of SCL did not influence the cell's sensitivity to the drug compared with its wild-type counterparts (data not shown). Many selenoproteins are involved in alleviating oxidative stress or have redox properties, for example, the glutathione peroxidases [10]. Perhaps selenoproteins in *T. brucei* are only expressed after infection of their mammalian host, as a way to survive an oxidative burst.

All Nfs-like proteins are known to contain both CysD and SCL activities [28]. Group I Nfs-like proteins (Nfs1, IscS and Nfs in this study) typically have approximately eightfold higher activity towards selenocysteine than cysteine. The preference for selenocysteine is much greater in Group II Nfs-like proteins (CpNifS, SufS and SCL in this study), where the activity can be up to 3000-fold higher towards selenocysteine [16,19]. Therefore, the interchangeable activities of SCL and Nfs of *T. brucei* are not surprising. It is worth noting that, whereas in the *T. brucei* procyclics downregulation of Nfs leads to a concomitant decrease in its binding partner IscU (P. Changmai & J. Lukeš, unpublished results), the level of IscU is not altered in cells depleted for SCL, indicating that there is no mutual dependence between these two proteins. Using specific antibodies against Nfs and α-TAP antibodies for the tagged SCL, we have shown that the former protein is confined to the mitochondrion, whereas the latter is, quite surprisingly, present mostly in the nucleus and cytoplasm. Thus, we have anticipated that upon downregulation of one of these enzymes, the CysD and SCL activities will decrease only in the compartment where the ablated protein resides. However, downregulation of SCL leads to a decrease in both activities in the cytosol and the mitochondrion, and a similar result was found for cells in which Nfs was targeted. Because the selected RNAi strategy and northern analysis ruled out possible off-target RNAi silencing, another explanation has to be put forward. It is possible that despite their immunoreactivity in only a single compartment, both proteins are also present at amounts undetectable with the available antibodies in the other cellular compartment, namely SCL in the mitochondrion and Nfs in the cytosol. Such a dual localization is known for Nfs1 in yeast, where the bulk of the enzyme resides in the organelle, but a small amount is also active in the nucleus [15]. Because there is only a faint signal [35], human CysD was initially overlooked in the nucleus. Recent identification of its binding partner Isd11 in this compartment, as well as in the mitochondrion, speaks in favor of a dual (or even multiple) localization of numerous Fe–S cluster assembly proteins in the eukaryotic cell [36].

Indeed, to explain the measured activities and their downregulation in respective RNAi knockdowns of *T. brucei*, such a dual localization of CysD and SCL can be invoked. However, the amounts of both proteins in the 'other' compartment must be very small, because neither the polyclonal antibody against Nfs, nor tagging of SCL allowed detection of the respective proteins in the cytosol and mitochondrion. Alternatively, indirect secondary effects may explain the observed activity profiles. Nfs downregulation leads to a strong pleiotropic phenotype which may in turn result in a reduction of cytosolic SCL and CysD activities. By contrast, downregulation of SCL does not lead to an observable phenotype and the effects on mitochondrial enzyme activities are not as pronounced as the effect of Nfs ablation on cytosolic activities. The simultaneous loss of activities in both cytosolic and mitochondrial compartments may also be a reflection of some kind of coordination between cytosolic and mitochondrial Fe–S assembly machineries. RNAi-induced knockdown of either SCL or Nfs decreases

both activities in the *T. brucei* procyclics. One important difference between these RNAi cell lines is that although knockdown of SCL shows no growth phenotype, downregulation of Nfs substantially slows the growth of *T. brucei*, suggesting that it is the main Nfs protein in these flagellates. However, based on the available data, the growth phenotype of the Nfs knockdown can be ascribed to another function of this protein. The absence of Nfs disrupts Fe–S cluster assembly, monitored by the decrease in the activities of Fe–S cluster-containing proteins, such as the cytosolic and mitochondrial aconitases [20]. At the same time, a general decrease in tRNA thiolation affects their stability and surprisingly acts as a negative determinant for cytosine to uridine editing of mitochondrial $tRNA^{Trp}$, inevitably leading to disruption of mitochondrial translation [21]. It thus appears that it is primarily the lack of thiolation which causes the growth phenotype of procyclic *T. brucei* interfered against Nfs, because a similar decrease in CysD activity in the SCL RNAi cells is insufficient to markedly slow their growth. Consequently, it appears that in the absence of one Nfs-type enzyme in a given cellular compartment, the other Nfs-type protein or another as yet unknown protein with an overlapping activity upholds the CysD and SCL activities at a level sufficient for survival, although at levels significantly lower than those in the wild-type cells. This is not particularly surprising in the case of mitochondrial and cytosolic SCL activities, which remain relatively high in both knockdowns. However, it is quite unexpected in the case of mitochondrial CysD activity, which decreases in the SCL knockdowns to only $\sim 15\%$ of the wild-type level, yet the cells are still able to retain unabated growth.

Using MS analysis we have shown that, like in other eukaryotes, *T. brucei* Nfs co-purifies with its highly conserved binding partner Isd11 (Z Paris, P Changmai & J Lukeš unpublished results), although SCL does not seem to stably interact with any other protein (this study). However, SCL is still capable of strong CysD activity *in vitro*, although the same activity of the Nfs protein in microsporidia was shown to be strongly potentiated by bound Isd11 [4], the knockdown of which is lethal in yeast [2,3] as well as in trypanosomes (Z Paris, P Changmai & J Lukeš, unpublished results). In *E. coli*, deletion of one Nfs-like protein is not lethal, which was attributed to complementation by another Nfs-like protein, SufS. We propose that in a similar fashion, Nfs can fully complement SCL, however, SCL can only partially fulfill the functions of Nfs, perhaps because it is incapable of binding Isd11.

Experimental procedures

Phylogenetic analysis

Available homologues for genes encoding Nfs/IscS, NifS and SCL from prokaryotes and eukaryotes were downloaded from GeneBank™. Special attention was placed on using genes in which the function in question had been confirmed experimentally. Amino acid sequences of the genes were aligned using KALIGN [37]; ambiguously aligned regions and gaps were excluded from further analysis. Phylogenetic trees were computed using maximum likelihood (PHYML) [38], maximum parsimony (PAUP* b4.10) [39] and neighbor joining (ASATURA; the particular method is designed to deal with saturation of amino acid positions) methods [40]. The model for amino acid substitutions (WAG + I + Γ) was inferred from the dataset using PROTTEST [41]. Analogously, all parameters for maximum likelihood analysis (likelihood of the tree is ln = −53614.118606; gamma shape parameter = 1.249; proportion of invariants = 0.012) were derived from the particular dataset. The robustness of constructed trees was tested by bootstrap analyses (maximum likelihood in 300 replicates; maximum parsimony and neighbor joining with 1000 replicates) and is indicated in Fig. 1. Both *T. brucei* genes are highlighted.

Plasmid constructs, transfection, RNAi induction and growth curves

The *T. brucei* procyclic cell lines with inducible ablation of either Nfs or Nfs-like protein were described previously [20,21]. Synthesis of double-stranded RNA was induced by the addition of 1 $\mu g \cdot mL^{-1}$ tetracycline. Two clonal cell lines (A and D), in which the Nfs-like mRNA was targeted, were obtained by limiting dilution in plates at 27 °C in the presence of 5% CO_2. An HA_3-tagged Nfs-like fusion protein expressed from the pJH54 vector was electroporated into the 29-13 procyclics as described elsewhere [21]. Next, mitochondrial and cytosolic fractions were obtained from cells resistant to 1 $\mu g \cdot mL^{-1}$ tetracycline and used for immunodetection of the HA_3-tagged protein.

Northern and western blots

Detection of Nfs-like mRNA isolated from the noninduced cells and cells 2, 4 and 6 days of RNAi induction was carried by northern blot analysis using a random primed labeled probe and formaldehyde gel electrophoresis of total RNA following standard protocols [42]. All antibodies used for western blots were generated against *T. brucei* proteins overexpressed in *E. coli*. Cell lysates corresponding to 5×10^6 procyclic cells per lane were separated on a 12% SDS/PAGE gel and blotted. The polyclonal rabbit antibodies against IscU, MRP2, GAP1, PHB1 and enolase were used at 1 : 1000, 1 : 1000, 1 : 1000, 1 : 1000 and 1 : 150 000, respec-

tively [43–45]. The polyclonal chicken antibodies against Nfs were used at 1 : 500. Secondary anti-rabbit IgG (1 : 1000) (Sevapharma, Prague, Czech Republic) coupled to horseradish peroxidase were visualized using the ECL kit (Amersham Biosciences, Uppsala, Sweden). To detect the Nfs-like protein, lysates from cells stably expressing the SCL protein HA_3-tagged at its C-terminus were separated and blotted as described above, and the membranes were treated with monoclonal anti-HA_3-tag mouse IgG, followed by chicken anti-mouse IgG coupled to horseradish peroxidase. Western blot bands were quantified with the software LUMINESCENT IMAGE ANALZYER LAS-3000, Image Reader LAS-3000.

TAP-tag analysis

The whole Nfs-like gene was PCR amplified and cloned into pLew79–MHT vector which contains c-*myc*, His, calmodulin-binding peptide and protein A tags in that order. The last two tags are separated by a TEV protease cleavage site [46]. Upon linearization by *Not*I, the resulting construct was transfected into the *T. brucei* 29-13 procyclic strain. Nfs-like TAP cells, checked for inducible and tightly regulated expression, were induced for 48 h by the addition of 1 $\mu g \cdot mL^{-1}$ of tetracycline to the medium. TAP purification was performed as described elsewhere [43].

Digitonin fractionation and subcellular localization

Purification of mitochondrial vesicles isolated by digitonin fractionation from 5×10^8 cells was performed as described elsewhere [21]. Pelleted mitochondrial vesicles were stored at −80 °C until further use. Subcellular localization of the expressed tagged protein within the cell was determined by immunofluorescence assay using polyclonal anti-Myc IgG (Invitrogen, Carlsbad, CA, USA). Briefly the cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% fetal bovine serum, and incubated with anti-Myc IgG at a 1 : 100 dilution. After washing, the cells were incubated with anti-rabbit fluorescein isothiocyanate-conjugated IgG (1 : 250 dilution) (Sigma, Steinheim, Germany), washed, and treated with 4′,6-diamidino-2-phenylindole stain to visualize DNA. Co-localization analysis was performed using mAb56 against the mitochondrial MRP1/MRP2 complex [27] coupled with Texas Red-X conjugated secondary antibody (Invitrogen). Phase-contrast images of the cells and their fluorescence were captured with a Nikon fluorescence microscope equipped with a camera and appropriate filters.

Enzyme essays

Cysteine desulfurase activity was assayed at 25 °C, essentially as described previously [47]. Briefly, protein extract was added to a reaction mixture containing 25 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 μM pyridoxal 5′-phosphate, 1 mM dithiothreitol and 500 μM cysteine. The reaction was stopped by the addition of 20 μL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine in 7.2 M HCl. Methylene blue was formed by the addition of 20 μL of 30 mM $FeCl_3$ in 1.2 M HCl and was assayed by measuring the absorbance at 670 nm. The selenocysteine lyase activity was measured as described elsewhere [31]. In short, a 100 μL reaction mixture of 0.12 M tricine, 10 mM selenocysteine, 50 mM dithiothreitol and 0.2 mM pyridoxal phosphate was allowed to incubate for 30 min, before being stopped with lead acetate. The formation of lead–selenide was quantified at 400 nm.

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