The Effect of RNA Interference Down-regulation of RNA Editing 3'-Terminal Uridylyl Transferase (TUTase) 1 on Mitochondrial de Novo Protein Synthesis and Stability of Respiratory Complexes in Trypanosoma brucei*

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Inhibition of RNA editing by down-regulation of expression of the mitochondrial RNA editing TUTase 1 by RNA interference had profound effects on kinetoplast biogenesis in Trypanosoma brucei procyclic cells. De novo synthesis of the apocytochrome b and cytochrome oxidase subunit I proteins was no longer detectable after 3 days of RNAi. The effect on protein synthesis correlated with a decline in the levels of the assembled mitochondrial respiratory complexes III and IV, and also cyanide-sensitive oxygen uptake. The steady-state levels of nuclear-encoded subunits of complexes III and IV were also significantly decreased. Because the levels of the corresponding mRNAs were not affected, the observed effect was likely due to an increased turnover of these imported mitochondrial proteins. This induced protein degradation was selective for components of complexes III and IV, because little effect was observed on components of the F₁,F₀-ATPase complex and on several other mitochondrial proteins.

Gene expression in the kinetoplast-mitochondrion of trypanosomatid protists involves a unique post-transcriptional mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript, during the mRNA maturation process, termed RNA editing, whereby U-residues are inserted to or deleted from a pre-edited transcript.

EXPERIMENTAL PROCEDURES

Cell Cultivation and RNAi—Procyclic cells of the T. brucei strain 29–13 were grown at 27 °C in SDM-79 medium supplemented with 10% bovine serum albumin (20). The cell line stably transfected with the RET1 expression construct (pTUTi-H2H) was derived previously (16). RNAi was induced with 1 μg/ml tetracycline. Induced cells were maintained in log-phase growth for 10 days.

Polypeptide Synthesis in Whole Cells—The procedures were described previously (22). Cells (10⁶) were pelleted by centrifugation at 16,000 × g for 5 min and washed with SoTe buffer (0.6 mM sorbitol, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA). Labeling of ~10⁶ cells was performed using EasyTag® EXPRESS®-S³-S³ protein labeling mix (PerkinElmer Life Sciences, >1000 Ci/mmol) at 100 μCi per 100-μl reaction in the presence of 100 μg/ml cycloheximide and 100 μg/ml other antibiotics as specified below for 2 h at 27 °C.

Electrophoretic, Autoradiographic, and Immunochronical Procedures—Samples were analyzed by single-dimension Tris-glycine SDS-
polycrylamide gels (23), two-dimensional Tris-glycine SDS gels (24), and two-dimensional Blue Native/Tricine SDS-polycrylamide gels (25). The resolved polypeptides were transferred onto nitrocellulose membranes by semidyblotting, as described previously (19). After electrophoresis, the gels were stained either with Coomassie Brilliant Blue R250 (Sigma) or SYPRO Ruby (Molecular Probes). For autoradiography, dried gels were exposed to low energy screens and analyzed by using the PhosphorImager (Molecular Dynamics).

Mouse antibodies (Oxone Research Products) were raised against gel-purified subunit XI (trCOXI) or band 8 of cytochrome c oxidase and Rieske iron-sulfur protein of cytochrome bc1 from L. tarentolae. The complexes were isolated according to our earlier procedure (26). The rabbit antisera against subunits I and IV of cytochrome c oxidase were described earlier (19, 27). The antisera against F1 and S9 of F1-F0-ATPase (28) were provided by N. Williams. The antisera against the p18 protein (subunit b of ATPase) was described previously (29). The antisera against cytochrome c1 (30) was provided by S. Hajduk. The antisera against the T. brucei TAO (31) and hsp60 (32) proteins were provided by G. Hill and F. Bringaud, respectively. The antiserum generated against subunits I and IV of cytochrome c oxidase were described previously (34) using the following mRNA-specific antisense oligonucleotides: 3807, 5’-GTTGATCGGCCATCGTAAATCAAGTGGATG-3’ (cytochrome c); 5’-ACGGACCTGATCCTGCGAC-3’ (apocytochrome b, Cyb; 5’-TTCCTCTAG-3’ Rieske protein); 5’-CAACCTGACATTAAAAGAC-3’ (apoapocytochrome b, Cyb); 5’-ACGGGCGGTGTTCCTTTGATG-3’ (apoapocytochrome c1); 5’-CGGACGCTGACTTTCCGAG-3’ (apoapocytochrome c); 5’-CAACCTGACATTAAAAGAC-3’ (apoapocytochrome b, Cyb); 5’-ACGGGCGGTGTTCCTTTGATG-3’ (apoapocytochrome c1) (trCOXI) COI; 5’-ACGAGGCGCGGTGTTCCTTTGATG-3’ (trCOII) COI.

RESULTS

Mitochondrial Cyb and COI Proteins Can Be Labeled by Incubation of Intact Cells with [35S]Methionine Plus Cysteine in the Presence of Cycloheximide—It was shown previously that two of the maxicircle DNA-encoded proteins from L. tarentolae mitochondria are detectable by two-dimensional SDS gel electrophoresis as spots with a characteristic position off the main diagonal due to the extremely high hydrophobicity of these proteins (18, 19). A similar separation was seen using T. brucei mitochondria (Fig. 1A). The peptide, FAFYCOR, was obtained by trypsin digestion of the spot labeled “Cyb”; this sequence matched positions 206–210 of the T. brucei apocytochrome b polypeptide sequence (38), confirming that this spot represented monomeric Cyb, as is the case in L. tarentolae. This peptide was also detected from a digest of the spot labeled “Cyb/tub”; however, a peptide from α-tubulin was detected in addition to several unidentified peptides, indicating that this represented aggregated material which included Cyb (18).

The COI polypeptide from an L. tarentolae mitochondrial extract migrated as a discrete stained spot off the diagonal, close to the monomeric Cyb polypeptide; however, in T. brucei mitochondrial extract, the COI spot was not visible. The faint stained spots labeled a and b in Fig. 1A did not contain COI-derived peptides, but rather represented contamination with several non-mitochondrial T. brucei proteins (data not shown). This is not unexpected, because the COI polypeptide from L. tarentolae was found previously to be refractory to trypsin digestion,3 and a similar property is anticipated for the T. brucei protein.

Both COI and Cyb could, however, be detected by incubation of intact cells for two hours with [35S]methionine plus cysteine in the presence of cycloheximide and followed by electrophoresis in a 9.5%/14% two-dimensional denaturing gel (Fig. 1B). The labeled spot “Cyb” in Fig. 1B corresponded to the stained Cyb spot in Fig. 1A, and the spot “COI” migrated in the same relative position as the COI spot in L. tarentolae. The identity

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2 Protein Prospector is available on the internet at prospector.ucsf.edu, and the parasite genome database is available at www.genedb.org.

3 A. Horváth and D. A. Maslov, unpublished observations.
of the Cyb and COI spots were further substantiated by showing sensitivity of the in vivo incorporation of labeled amino acids to puromycin (Fig. 2) and insensitivity to chloramphenicol, tetracycline, hygromycin B, as was previously found in organello mitochondrial protein synthesis in L. tarentolae (22). This method for in vivo labeling and detection of the mitochondrial Cyb and COI proteins represents a valuable tool to study mitochondrial protein synthesis in these cells.

Mitochondrial Biosynthesis of COI and Cyb Proteins Decreases during RET1 Silencing—It was shown previously that the RET1 TUTase is essential for viability of procyclic T. brucei (16). Down-regulation of expression of RET1 by conditional RNAi affected editing of maxicircle-derived mRNAs (16) by affecting the length of the guide RNA 3′ oligo-U tails (37). It follows that RET1 RNAi should also affect synthesis of proteins such as Cyb, where the mRNAs require editing to be translatable. We examined this possibility by assaying the in vivo labeling of Cyb at different times after induction of RNAi with tetracycline. In vivo labeling of the COI protein, which is translated from a never-edited mRNA, was also followed. Aliquots of the culture, the growth of which is shown in Fig. 3A, were withdrawn on days 0, 1, 2, 3, and 6 after induction, and the cells were labeled for two hours with 35S-amino acids in the presence of cycloheximide, followed by two-dimensional gel electrophoresis to visualize the labeled Cyb and COI spots (Fig. 3B). The synthesis of Cyb was reduced by day 2 and was not detectable at day 3 after RNAi induction. Surprisingly, the synthesis of COI also decreased by approximately the same extent. The extent of decrease in synthesis of Cyb and COI by day 3 in several independent RET1-silencing experiments varied from 70–100%. No effect was observed by treating the parental 29–13 cells with the same concentration of tetracycline (not shown).

The Levels of Several Nuclear-encoded Subunits of Respiratory Complexes III and IV Also Decrease upon RET1 Silencing—Mitochondrial fractions were isolated on days 0, 3, 5 and 7 after RET1 RNAi induction and the relative abundances of several mitochondrial proteins were analyzed by Western blotting (Fig. 4). The uppermost control panel shows the expected decrease in the RET1 protein by day 3. Mitochondrial matrix proteins, the RNA-binding protein, gBP21 (originally p28) (9), and hsp60 (32), and the inner membrane-localized alternative oxidase (TAO; Refs. 31, 38) showed little change until day 7, by which time RET1-silenced cells usually display abnormal morphology (16). The nuclear-encoded trCOIV and trCOXI subunits of respiratory complex IV showed a dramatic decrease in abundance by day 3 and were undetectable by day 5. Two nuclear-encoded components of respiratory complex III, the Rieske iron-sulfur protein and cytochrome c1, showed similar decreases in steady-state abundance. On the other hand, subunits β, b (originally p18; Ref. 29), and S9 of F1/F0-ATPase remained stable or even slightly increased in relative abundance.

The Assembly of Respiratory Complexes III and IV, but Not Complex V, Is Affected by RET1 Silencing—The effect of RET1 silencing on the assembly of the respiratory complexes was analyzed by the Blue Native/Tricine-SDS two-dimensional gel electrophoresis method which was used previously to identify respiratory complexes in L. tarentolae, T. brucei, and Leishmania amazonensis (27). The use of a 6% gel in the first dimension (Fig. 5A) allowed a well defined separation of complexes IV and III. However, a gradient gel (3–13%; Fig. 5B) allowed for a substantially better separation of monomeric and oligomeric forms of the F1/F0-ATPase complex V. We performed the silencing analysis with both gel systems, but only the gradient gels are presented in Fig. 5B. The relative abundances of complexes III, IV, and the oligomeric forms of complex V decreased strongly during the RET1 silencing and were undetectable at day 5. Only the monomeric form of complex V (Fig. 5, B–E, V′) remained relatively unaffected during the silencing.

The decrease of several specific subunits of complex III and complex IV by day 3 of silencing is shown in the Western blots in Fig. 5, C–E. Interestingly, there was no accumulation of proteins migrating at positions of partially assembled or degraded complexes, suggesting that degradation of the complexes caused by the silencing of RET1 must be rapid and complete (not shown).

Western analysis of the ATPase complex V in Fig. 5E confirms that this complex contains the F0 as well as the F1 moiety of the ATPase complex, and shows that the abundance of the oligomeric forms is reduced, whereas the abundance of the monomeric form is increased, as was observed in the stained gels in Fig. 5B.

RET1 Silencing Has No Effect on Nuclear-encoded mRNAs for Respiratory Complex Subunits—Down-regulation of RET1 expression was shown previously to inhibit RNA editing but to
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Fig. 3. Analysis of translation products in T. brucei RET1 RNAi-induced cells. A, growth curve of T. brucei cells after RET1 RNAi induction. O, uninduced cells; •, tetracycline-induced cells. Cells were diluted daily to maintain log-phase growth. B, cells harvested at different times after induction of RNAi were labeled for 2 h with EXPRESS®35S labeling mix (PerkinElmer Life Sciences) in the presence of 100 μg/ml cycloheximide, and the products were fractionated in 9.5%/14% two-dimensional SDS gels. Inset panels, the corresponding Coomassie Brilliant Blue R250-stained gels. The time of induction is shown above each panel.

Fig. 4. Effect of RET1 silencing on several representative nuclear-encoded mitochondrial proteins. Mitochondrial extracts from T. brucei cells harvested on the indicated days after RET1 RNAi induction were analyzed by Western blotting. The antiserum used for each panel is shown on the right.
have no effect on transcription or turn-over of mitochondrial-encoded mRNAs for either edited or never-edited genes (16). Because we have observed a decrease in the abundance of nuclear-encoded subunits of respiratory complexes III and IV, we investigated whether this was caused by a decrease in the corresponding mRNAs or an increased turn-over of the proteins. Total cell RNA from RET1-silenced cells was isolated at days 0, 1, 3, and 5 after RNAi induction, and the relative abundances of several specific mRNAs were analyzed by using a primer extension assay (Fig. 6). Calmodulin mRNA, which encodes a non-mitochondrial protein, and TAO mRNA, which encodes a cytosolic-synthesized mitochondrial protein not present in respiratory complexes III and IV, were used for internal normalization of the assays. The steady-state levels of Rieske, cytochrome c1, and trCOIV mRNAs remained essentially unaffected by silencing of RET1 (Fig. 6). These results suggest that the decrease in abundance of the nuclear-encoded subunits of complexes III and IV is due to increased turn-over, probably as a result of an effect on assembly or stability of these complexes caused by the decrease in the mitochondrial-encoded subunits.

Changes in Cyanide-sensitive Respiration of RET1-silenced Cells—Two modes of oxygen consumption are observed in procyclic T. brucei: cyanide-sensitive respiration mediated by respiratory complex IV and salicylhydroxamic acid-sensitive/cyanide-insensitive respiration mediated by trypanosome alternate oxidase (reviewed in Ref. 39). We examined the effect of RET1 silencing on these two modes of respiration. Control cells showed an oxygen uptake rate of 4.7 ± 2.6 × 10⁻¹⁰ μmol/cell/min, of which ~80% represents cyanide-sensitive respiration. By day 3 of RET1 silencing, the rate of oxygen consumption decreased to ~50% of the control level, mainly as a result of a selective decrease in cyanide-sensitive respiration (Fig. 7). Oxygen uptake by the alternate oxidase pathway was not affected. These results are consistent with the above data.
on the effect of down-regulation of RET1 expression on the assembly or stability of respiratory complexes and the lack of effect on the level of the alternative oxidase.

**DISCUSSION**

The results in this paper have shed some light on the interactions of editing and translation in the kinetoplast-mitochondrion of trypanosomatid protozoa. Conditional down-regulation of RET1 expression in procyclic *T. brucei* by RNAi was shown previously to be lethal (16). The role of RET1 was shown to involve the addition of untemplated U’s to the 3’ ends of guide RNAs, thereby creating a 3’ oligo-U tail, which is apparently required for editing (37). In this paper, we have presented evidence that the effect on viability is due, at least in part, to an effect on the assembly or stability of the cytochrome bc1 complex (respiratory complex III) and the cytochrome c oxidase complex (respiratory complex IV), both of which contain some subunits translated from edited mRNAs. Similar results were obtained with conditional down-regulation of RET2 synthesis (data not shown).

A rapid inhibition of mitochondrial protein synthesis of the Cyb protein, which is encoded by an edited mRNA, was demonstrated by development of a method to pulse-label newly synthesized mitochondrial proteins in intact *T. brucei* cells. This result provides additional evidence that edited mRNAs are translated and that the translated proteins are required for mitochondrial function.

A rapid decrease in the steady-state abundance of several nuclear-encoded subunits of these respiratory complexes was also observed. Because there was no effect on the steady-state levels of the mRNAs, the observed decreases in abundance were probably due to increased turnover of the proteins. We realize that down-regulation of expression of RET1 by RNAi is not the best experimental method to obtain data on protein turnover, because of uncertainties involving the stability of the mRNA and the protein itself both free and incorporated in the pre-existing complex. We attempted to measure *in vivo* turnover of Rieske and COIV directly by a pulse-chase experiment using specific antisera, but this was unsuccessful for technical reasons.

There is, however, sufficient precedent in other systems for the rapid degradation of mitochondrial proteins not incorporated into a respiratory complex. In yeast with defective mitochondrial protein synthesis, nuclear-encoded subunits of respiratory complexes III and IV continue to be synthesized and imported into the mitochondrion, but assembly of the complexes is impaired (40). In addition, a specific nuclear mutation (*PET309*) affecting the synthesis of Cox1p in yeast resulted in an increased proteolytic degradation of other mitochondrial and nuclear subunits (41, 42); interference with binding of mRNA-specific activators (43, 44) prevented membrane tethering of these mRNAs and induced increased turn-over of the newly synthesized polypeptides (45). In mammalian cells in which mitochondrial protein synthesis was blocked by ethidium bromide, the synthesis of nuclear-encoded subunits of complexes I, III, and IV was not affected, but the proteins were rapidly degraded (46, 47).

We hypothesize that in the case of RET1-down-regulated *T. brucei*, the absence of the newly synthesized mitochondrial subunits of complexes III and IV is responsible for the breakdown of the respiratory complexes and the subsequent rapid turn-over of imported nuclear-encoded subunits. The mechanism by which the silencing of RET1 results in a decreased level of the *de novo* synthesized mitochondrial polypeptides still needs to be elucidated. The Cyb protein is translated from a 5’ edited maxicircle-encoded mRNA; therefore, mitochondrial translation of Cyb would be directly affected because of the effect on RNA editing. The COI protein, however, is translated from a unedited maxicircle-encoded mRNA, the level of which is unaffected by RET1 silencing. This suggests that the observed effect may be due to an increased turnover of newly synthesized translation products. Little is known at present about these aspects of mitochondrial gene expression in trypanosomes.

Interestingly, RET1 silencing had no effect on the abundance of the nuclear-encoded subunits of F1, nor of at least one nuclear-encoded component of F0. Nor was there any effect on several other mitochondrial proteins, including the alternative oxidase, a heat shock protein, and an RNA-binding protein possibly involved in RNA editing. This is consistent with results in other systems which showed that the ATPase complex is the least sensitive to the inhibition of mitochondrial protein synthesis as compared with other respiratory complexes (46). It was, however, somewhat surprising that assembly of F1-F0-ATPase in *T. brucei* was apparently unaffected by the inhibition of mitochondrial protein synthesis by edited mRNAs, because a pan-edited maxicircle gene was thought to represent a homologue of subunit 6 of F1-F0-ATPase (48, 49). However, direct evidence for the A6 protein actually being a functional subunit of F0 in *T. brucei* is lacking, and a resolution of this problem awaits further investigation.

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