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An RNA-binding Respiratory Component Mediates Import of Type II tRNAs into *Leishmania* Mitochondria*[S]

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Transport of tRNAs across the inner mitochondrial membrane of the kinetoplastid protozoon *Leishmania* requires interactions with specific binding proteins (receptors) in a multi-subunit complex. The allosteric model of import regulation proposes cooperative and antagonistic interactions between two or more receptors with binding specificities for distinct tRNA families (types I and II, respectively). To identify the type II receptor, the gene encoding RIC8A, a subunit of the complex, was cloned. The C-terminal region of RIC8A is homologous to subunit 6b of ubiquinol cytochrome *c* reductase (respiratory complex III), while the N-terminal region has intrinsic affinity for type II, but not for type I, tRNAs. RIC8A is shared by the import complex and complex III, indicating its bi-functionality, but is assembled differently in the two complexes. Knockdown of RIC8A in *Leishmania* lowered the mitochondrial content of type II tRNAs but raised that of type I tRNAs, with downstream effects on mitochondrial translation and respiration, and cell death. In RIC8A knockdown cells, a subcomplex was formed that interacted with type I tRNA, but the negative regulation by type II tRNA was lost. Mitochondrial extracts from these cells were defective for type II, but not type I, import; import and regulation were restored by purified RIC8A. These results provide evidence for the relevance of allosteric regulation *in vivo* and indicate that acquisition of new tRNA-binding domains by ancient respiratory components have played a key role in the evolution of mitochondrial tRNA import.

Mitochondria from a large number of species, including protists, higher plants, some invertebrates, and eutherian mammals do not contain sufficient numbers of functional tRNA genes and therefore import cytoplasmic tRNAs to support the translation of organellar mRNAs (reviewed in Refs. 1 and 2). Mitochondrial tRNA import is especially important in kinetoplastid protozoa such as *Leishmania* and *Trypanosoma* that lack all mitochondrial tRNA genes (3, 4).

The precise manner in which polyanionic tRNA molecules cross the double mitochondrial membrane, is largely unresolved. In yeast, the mitochondrial protein import pore, as well as cytosolic carrier proteins, appears to be involved (5, 6). On the other hand, biochemical studies in kinetoplastid protozoa have revealed that these organisms have specialized mechanisms for import of tRNA that are distinct from those for protein import (7) but involve direct interaction of tRNAs with membrane-bound proteins (8). *Leishmania* and *Trypanosoma* mitochondria recognize sequence/structure motifs (import signals) in distinct domains of individual tRNAs (9, 10), and the former rapidly select oligoribonucleotide-containing motifs matching those in importable tRNAs from a random sequence pool of high complexity (11).

However, there are differences in the intrinsic efficiencies of transfer of individual RNAs through the inner, as opposed to the outer membrane, *in vitro*; some, designated as type I RNAs, are imported efficiently into the matrix, whereas others (type II) are not. The potential problem of an imbalanced matrix tRNA pool is solved by the unique phenomenon of allosteric regulation; type I RNAs stimulate the inner membrane transfer of type II RNAs, whereas type II RNAs inhibit transfer of type I (11, 12). On the basis of these interactions, $tRNA^{Tyr}$, $tRNA^{Arg}$, and $tRNA^{Trp}$ have been identified as type I, and $tRNA^{Ile}$, $tRNA^{Val}$, and $tRNA^{Met\text{-}e}$ have been identified as type II, respectively (11–13); *in vitro* evolution experiments suggest that many more tRNA species belong to these categories (11). The "ping-pong" model (14) postulates that the two types of tRNA bind to different receptors; binding of type I tRNA to its receptor induces a conformational change that is transmitted to the type II receptor, opening up its tRNA-binding site. Type II tRNA loading in turn induces an allosteric transition resulting in the destabilization of the type I complex.

Recently, a multi-protein complex (the RNA import complex (RIC))[2] that induces translocation of tRNAs through artificial or biological membranes was isolated from *Leishmania* inner membrane (14–16). In a reconstituted liposome system, RIC retains the characteristic type I-type II interactions (14). Two tRNA-binding proteins with the properties of type I and type II receptors are associated with this complex (14). We have recently identified the type I receptor and showed that, *in vivo*, it is required for import of type I as well as type II tRNAs (13).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and supplemental Figs. S1 and S2.

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[2] The abbreviations used are: RIC, RNA import complex; Tet, tetracycline; BN, Blue Native; RT, reverse transcription; ORF, open reading frame; UCR, ubiquinol cytochrome *c* reductase; BU, 5-bromouridine.

We now report the identification of a subunit of this complex that binds type II tRNAs and is essential for the import of these tRNAs *in vivo*.

MATERIALS AND METHODS

Sequence Analysis and Homology Modeling—Affinity-purified RNA Import Complex from *Leishmania tropica* inner mitochondrial membranes (14) was resolved into its subunits by SDS-PAGE. The Coomassie Blue-stained 21-kDa band of RIC8 was trypsinized, and the peptides were subjected to liquid chromatography/mass spectrometry and tandem mass spectrometry analysis at the W. M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia). Sequenced peptides were matched against the NCBI and *Leishmania major* data bases (www.genedb.org/genedb/leish/index.jsp) to identify the corresponding genes using the BLAST or Sequest program. Selected sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw/index.html). Secondary structure predictions were made by the PredictProtein server (www.cubic.bioc.columbia.edu/predictprotein/). Automated homology modeling of the *Leishmania* protein was performed by the SWISS-MODEL server (www.expasy.ch/swissmod/SWISS-MODEL.html), using the crystallographic structure of subunit 6 (Protein Data Bank code 1ntm) of bovine ubiquinol cytochrome *c* oxidase (Complex III) as template. The results were viewed in the Deep View/Swiss Pdb Viewer v. 3.7, and ray-traced images were generated using the POV-Ray 3.6 package.

Cloning of RIC8A Gene—Sense and antisense primers (supplemental Table S1) corresponding to the open reading frame of RIC8A were used to amplify the intact or truncated gene from *L. tropica* strain UR6 genomic DNA. The sense primer was designed to have a BamHI restriction site at the 5′ end in frame with the coding sequence of the insert; the antisense primer had an in-frame stop codon followed by a SalI site. The amplified fragment was cloned into TA vector pTZ57R (MBI Fermentas). Other genes were cloned and expressed similarly (13).

Preparation of Recombinant Proteins and Antibodies—The RIC8A open reading frame was transferred as a BamHI-EcoRI fragment from pTZ57R to the pGEX4T-1 vector (Amersham Biosciences) downstream of, and in-frame with, the glutathione *S*-transferase gene. Recombinant plasmids were expressed in *Escherichia coli* BL21, and fusion protein was extracted from inclusion bodies and digested with thrombin, and the recombinant protein was recovered by gel electrophoresis, as described (13). To refold the protein, the soluble SDS extract was diluted 5-fold into TETN250 buffer (14) containing 0.1% Triton X-100 and incubated for 2 h at 4 °C before assay. Electrophoretically pure recombinant protein was used to raise polyclonal antibody in BALB/c mice.

Blue Native (BN) PAGE—Inner membrane mitochondrial complexes of *L. tropica* or the human HepG2 cell line were resolved as previously described (13). Briefly, mitochondria were extracted with BAM buffer (50 mM BisTris-HCl, pH 7.0, 0.75 M ϵ-aminocaproic acid, 2% dodecyl maltoside) for 45 min at 4 °C, and the extract was concentrated to ~10 μl/200 μg of mitochondria by centrifugal ultrafiltration in a Microcon 30 unit (Amicon). Coomassie Blue G-250 (0.5%) was added to the extract before electrophoresis on 6% Blue Native gels (21). For two-dimensional analysis, protein bands of the first dimension were denatured with 0.125 M Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol for 40 min at 37 °C and then subjected to SDS-PAGE.

Preparation of Radiolabeled tRNA—^{32}P-Labeled tRNAs of high and low specific activity were prepared by T7 polymerase-mediated run-off transcription as described (11, 13).

Import Assays—Purified RIC (100 ng) was incorporated into phosphatidylcholine vesicles (50 μg lipid) and incubated with ^{32}P-labeled tRNA (5 nM) and 4 mM ATP, and uptake was analyzed by RNase protection, as previously described (14). For immuno-inhibition experiments, proteoliposomes were preincubated with antiserum (1:50) for 30 min on ice. Where indicated, low specific activity effector tRNAs were present at one-tenth the concentration of the high specific activity substrate tRNA.

Western and Northwestern Blots—Native complexes resolved by BN PAGE were denatured *in situ* before blot transfer, as described (13). The blots were probed with 1:100 dilution of antiserum and developed by the alkaline phosphatase colorimetric method. For Northwestern blots, the membrane was probed with ^{32}P-labeled tRNA (17).

Binding Assays—Indicated amounts of purified, recombinant refolded RIC8A were incubated with ^{32}P-labeled tRNA^{Ile} (10–100 fmol) in 10-μl reactions containing binding buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 M KCl) for 30 min on ice and then electrophoresed on native gradient polyacrylamide gels (14) to resolve the ribonucleoprotein from free tRNA. Dried gel bands were quantified by liquid scintillation counting. Liposome binding assays were carried out under similar conditions with 250 ng of purified RIC incorporated into 80 μg of lipid and 1–250 fmol of tRNA^{Ile}, the vesicles were washed, and bound RNA was recovered for electrophoresis. Scatchard analysis was carried out by titrating a fixed amount of RIC8A (50 fmol) or liposome-bound RIC with tRNA^{Ile}. The plot of bound/free tRNA against the bound tRNA concentration yields a best fit curve with a slope of $-1/K_d$, where K_d is the dissociation constant for the complex. The total receptor concentration, $[R]_0$, is the intercept on the *x* axis.

Photochemical Cross-linking—T7 RNA polymerase transcripts were doubly labeled with [α-^{32}P]UTP and 5-bromo-UTP and cross-linked to protein as previously described (14). Briefly, RNA was incubated with affinity-purified RIC or mitochondrial complexes and then UV-irradiated. Cross-linked RNA·protein complexes were immunoprecipitated after dissociation of the subunits with SDS and resolved by urea-PAGE (14).

Northern Blotting—Total promastigote RNA was electrophoresed on a 5% acrylamide, 8 M urea gel, electroblotted on to a Hybond N+ membrane, and probed with radiolabeled RIC8A coding region.

Conditional Knockdown—Details of the construction of *L. tropica* 13–90, a host containing constitutively expressed T7 RNA polymerase and Tet repressor genes, and of the targeting vector pGET, have been described (13). Knockdown vector pGET(AS)RIC8A was constructed by inserting the RIC8A gene between the HindIII and BamHI sites of pGET (*i.e.* in the

reverse orientation with respect to the inducible T7 promoter). *L. tropica* 13–90 was transfected with pGET(AS)RIC8A and transformants selected on semi-solid agar containing G418, hygromycin, and 2.5 μg/ml phleomycin. The clones were grown in medium 199 containing the same antibiotics. The cultures were induced with 1 μg/ml tetracycline, and cell growth was monitored. Intracellular parameters were measured at 48 h, the time point at which cessation of growth was first observed.

RT-PCR—Uninduced or induced cells were harvested, lysed, and separated into soluble (cytosolic) and particulate (mitochondrial) fractions (9). The crude mitochondria were treated with DNase and RNase before RNA isolation. RNA from 10^2–10^5 cells was denatured at 95 °C and reverse transcribed with Superscript II (Invitrogen) and the appropriate primer, as follows: (1) for antisense RNA, the sense primer from the 5′ end of the coding region of RIC8A; (2) for mRNA or tRNA, the antisense primer complementary to the 3′ end of the gene (supplemental Table S1). The second primer was then added, and the cDNA was amplified with *Taq* DNA polymerase. To obtain proportional PCR signals, RT-PCR was performed with different amounts of input RNA.

Import Reconstitution—Liposomes were reconstituted with mitochondrial extracts from knockdown cells and recombinant import factor as described (13). Briefly, refolded RIC8A (8 ng in 2 μl) and concentrated mitochondrial extract (5×10^6 cell equivalent in 8 μl) were incubated with liposomes (50 μg of lipid in 10 μl) for 1 h at 4 °C and then assayed for import as above.

RNA End Labeling—Total mitochondrial tRNA was dephosphorylated with shrimp alkaline phosphatase, ethanol-precipitated, and 5′-labeled with [γ-^{32}P] ATP in the presence of T4 polynucleotide kinase.

Mitochondrial Assays—Mitochondrial translation assays by [^{35}S]methionine labeling of promastogotes in presence of cycloheximide, oxygen uptake measurements, and cytochrome oxidase cytochemical assays were performed as described (13).

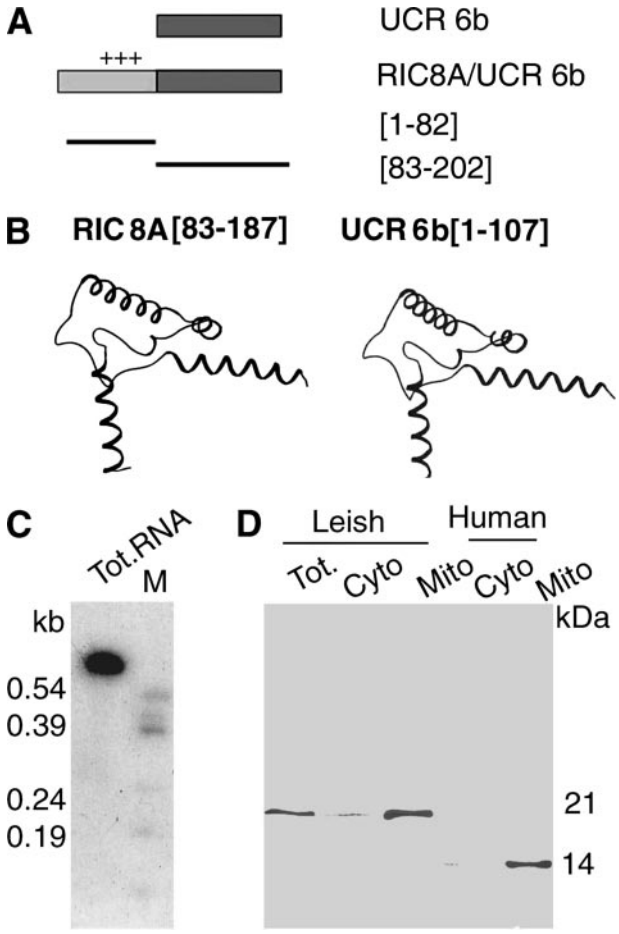

FIGURE 1. *A*, domains of *Leishmania* RIC8A (LmjF35.0100) compared with UCR6b from other organisms. The conserved region is *shaded black*, and the periodic repeat of six basic residues (+) at every third position is indicated. Sequence alignments are shown in supplemental Fig. S1. *B*, homology model of *Leishmania* RIC8A (*left panel*) based on the crystallographic structure of bovine UCR 6b (*right panel*). Only the peptide backbone of the homologous region is shown. *C*, Northern blot of *L. tropica* promastigote RNA using RIC8A probe. *M*, radiolabeled markers. *D*, intracellular distribution of RIC8A/UCR6b. Shown are immunoblots of total (*Tot.*), cytosolic (*Cyto*), or mitochondrial (*Mito*) protein (100 μg) from *L. tropica* or human (HepG2) cells, probed with RIC8A antiserum.

RESULTS

Homology of an RIC Subunit with a Complex III Component—On resolution of RIC by SDS-PAGE, a protein of 21 kDa (previously designated as RIC21p; now renamed RIC8) was observed that was present in the stoichiometry of 2–3/complex (Ref. 14; see Fig. 3*B*). This protein was subjected to mass spectrometric sequencing, and the tryptic peptides were searched for in the *L. major* data base (18) to retrieve complete open reading frames (ORFs). This resulted in the recovery of 10 ORFs, of which six were discarded as possible contaminants because of low protein coverage (less than 10%) or abnormally small size (less than 10 kDa). Of the remaining four (ORF-A, ORF-B, ORF-C, and ORF-D), ORF-C has sequence similarity with the iron-sulfur protein of mitochondrial succinate dehydrogenase (Complex II), ORF-B is a mitochondrial protein associated with a ribonucleoprotein (19), and ORF-D does not have sequence similarity with any known protein.[3] These four ORFs were expressed separately in *E. coli*, and antibody was raised against each recombinant protein. Of these four antibodies, only anti-RIC8A (ORF-A) inhibited import *in vitro*.[3] We therefore focused on the role of RIC8A, one of the major constituents of the 21-kDa band.

BLAST analysis of RIC8A (systematic gene name LmjF35.0100 in the *L. major* data base) shows sequence similarities (28–30% identity) to the subunit 6b (or in the case of yeast, subunit 7) of respiratory Complex III (ubiquinol cytochrome *c* reductase or UCR) from a wide variety of species (Fig. 1*A* and supplemental Fig. S1). The *L. tropica* coding region is identical to that from *L. major*.[3] In *Leishmania* and the related kinetoplastid protozoa *Trypanosoma cruzi* and *Trypanosoma brucei*, the corresponding predicted proteins are nearly identical in length (201–202 amino acid residues) and sequence (89 and 84% identity between *Leishmania* and the other two species). In other species such as man, UCR6b is smaller (14 kDa; see Fig. 1*D*). The sequence similarity between RIC8A and UCR6b is confined to the C-terminal 100 or so residues of the former. This similarity was confirmed by homology modeling

[3] S. Chatterjee and S. Adhya, unpublished data.

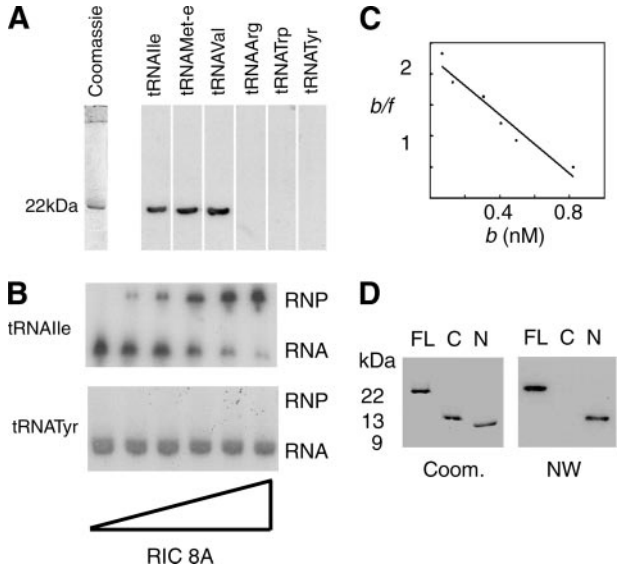

FIGURE 2. **Binding of type II tRNAs by RIC8A/UCR6b.** *A*, Northwestern blots of bacterially expressed and purified RIC8A/UCR6b (Coomassie stained at *left*) probed with ^{32}P-labeled tRNAs as indicated. The apparent molecular mass (22 kDa) of the expressed full-length protein, containing the mitochondrial targeting signal (Fig. S1), is higher than that of the mature 21-kDa RIC8A (Fig. 1*D*). *B*, gel shift assay showing titration of tRNA^{Ile} or tRNA^{Tyr} (10 fmol each) with 5, 10, 25, 50, 75, and 100 fmol of purified RIC8A. *C*, Scatchard plot of bound/free tRNA^{Ile} (*b/f*) *versus* bound tRNA^{Ile} concentration (*b*). *D*, tRNA binding activity of RIC8A domains. *Left panel*, Coomassie (*Coom.*) stain of bacterially expressed and purified full-length (*FL*) RIC8A, N-terminal residues 1–82 (*N*) and C-terminal residues 83–202 (*C*). *Right panel*, Northwestern (*NW*) blot of the same proteins probed with ^{32}P-labeled tRNA^{Ile}.

of the secondary structure (Fig. 1*B*), which shows a four-helical bundle in the C-terminal region of RIC8A that is superimposable on the crystallographic structure of UCR6 (20). The N-terminal extension does not show significant similarity to any known protein but is predicted to contain 1) several α-helices, one of which includes a periodic repeat of basic amino acids (between residues 42 and 61); and 2) a short cleaved mitochondrial targeting sequence, with the mature N terminus at Met-10 (as indicated by peptide sequencing).

In *Leishmania*, there is a single gene for this protein (LmjF35.0100; henceforth designated as RIC8A/UCR6b) on chromosome 35 (17). A single 0.77-kb mRNA is expressed in promastigotes (Fig. 1*C*), Moreover, a single 21-kDa protein is present predominantly in the mitochondrial fraction (Fig. 1*D*).

Type II tRNA Binding by RIC8A/UCR6b—To assess the tRNA binding activity of RIC8A, Western blots of bacterially expressed, gel-purified RIC8A, after suitable renaturation treatments *in situ*, were probed with radiolabeled tRNAs. Under these conditions, RIC8A interacted with tRNA^{Ile}, tRNA^{Val}, and tRNA$^{Met\text{-}e}$ (all type II tRNAs), but with none of the type I tRNAs (Fig. 2*A*). The tRNA·protein complex was detected by gel shift assays using purified RIC8A, which confirmed the specificity for type II tRNA (Fig. 2*B*). Scatchard analysis (Fig. 2*C*) yielded a dissociation constant (K_d) of 0.42 nM at 4 °C for the tRNA^{Ile}·RIC8A complex (Table 1). Thus, RIC8A has an intrinsic affinity for type II tRNAs.

To determine the location of the tRNA-binding site, the N- and C-terminal domains (residues 1–82 and 83–202, respectively) were separately expressed in *E. coli*, and the purified proteins were analyzed by Northwestern blotting using tRNA^{Ile} as probe. This experiment showed that only the N-terminal domain contains tRNA binding activity (Fig. 2*D*).

TABLE 1
Affinity of RIC8A for tRNA^{Ile}

Dissociation constants (K_d) and total receptor concentration ($[R]_0$) were estimated from Scatchard plots. Free, recombinant protein. Complex-bound, RIC incorporated into liposomes. Effector tRNA^{Tyr} was present at 0.1 nM.

Protein	tRNA^{Tyr} effector	K_d	$[R]_0$
		nM	nM
Free RIC8A	−	0.42	2.28
Complex-bound RIC8A	−	26.24	0.16
Complex-bound RIC8A	+	5.29	1.75

Presence of RIC8A/UCR6b in Two Mitochondrial Complexes—In view of the facts that RIC8A/UCR6b is the product of a single gene, has structural similarity with UCR6b, binds tRNA, and is associated with the import complex, it is possible that RIC8A/UCR6b is a bi-functional protein with roles in both tRNA import and electron transport. To address this question, we resolved the mitochondrial inner membrane respiratory complexes of *Leishmania* by Blue Native gel electrophoresis (21) (Fig. 3*A*, *left panel*). As observed by us and others in the kinetoplastid protozoa *Leishmania*, *Crithidia*, and *Phytomonas*, (13, 22–26), four complexes were discernible, corresponding to Complexes III, IV, and V and the largest complex, specific to kinetoplastid protozoa, that has been identified as the import complex (13). Immunoblotting of the resolved complexes revealed the presence of RIC8A/UCR6b in RIC and Complex III (Fig. 3*A*, *right panel*). The subunits of complex III and RIC were separated by denaturing electrophoresis in the second dimension. Most of the subunits in the two complexes are distinct, but Western blot analysis showed that the same 21-kDa RIC8A/UCR6b is shared by both (Fig. 3*B*), as expected of a bi-functional protein.

Because RIC8A/UCR6b is shared by RIC and Complex III, we inquired whether both complexes can bind tRNA. When the mixture of native respiratory complexes obtained by detergent extraction of mitochondria was exposed to tRNA^{Ile} (type II) doubly labeled with ^{32}P and 5-bromouridine (BU), a photoactivable nucleoside analogue, and then UV-irradiated, only RIC among the mitochondrial complexes was tagged; cross-linking required the presence of type I tRNA (Fig. 3*C*). In contrast, none of the human respiratory complexes interacted with tRNA under any condition (Fig. 3*C*). After dissociation of the complex formed with RIC, the single RNA-protein adduct could be resolved by denaturing PAGE and was immunoprecipitated with anti-RIC8A/UCR6b antibody; the antibody, if present during the tRNA binding reaction, prevented formation of the adduct (Fig. 3*D*). Identical results were obtained with BU-labeled tRNA^{Val} and tRNA^{Met}, two other type II tRNAs; nonimmune serum, or antibody against other RIC subunits, failed to immunoprecipitate this adduct.[3] Thus, RIC8A/UCR6b is the major, if not the sole, component of the import complex that directly interacts with type II tRNAs.

The allosteric activation of binding of the type II tRNA^{Ile} by type I tRNA^{Tyr} was quantified by binding assays on the liposome-bound import complex. This showed that the type I tRNA^{Tyr} increases the affinity, *i.e.* lowers the K_d, of tRNA^{Ile} for the complex by a factor of 5 (Table 1). Additionally, there is a

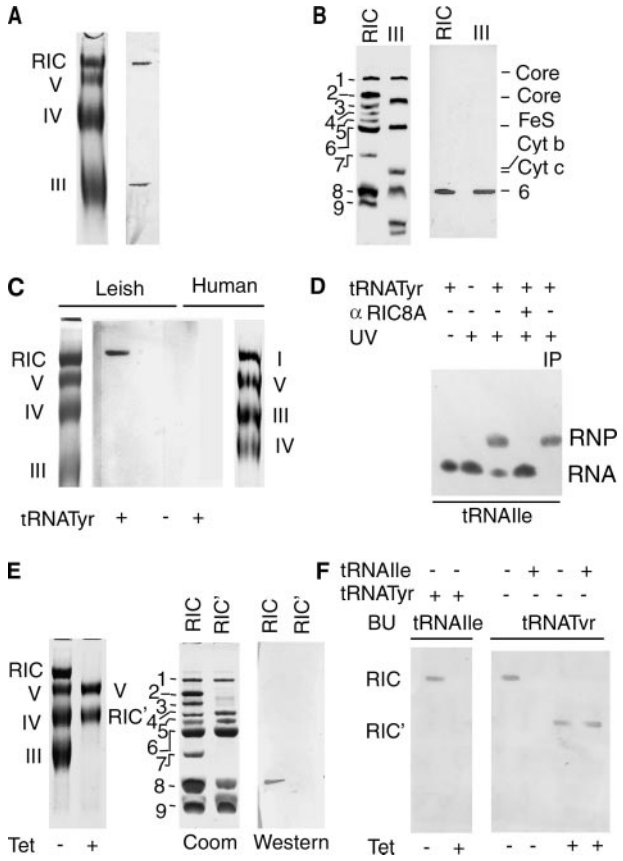

FIGURE 3. **Distribution of RIC8A/UCR6b in mitochondrial complexes.** *A*, *left panel*, Blue Native PAGE profile of *L. tropica* mitochondria. *Right panel*, immunoblot of the resolved complexes using anti-RIC8A antibody as probe. *B*, second dimension (SDS-PAGE) profile of RIC and Complex III. *Left panel*, Coomassie stain. *Right panel*, immunoblot with anti-RIC8A antibody. RIC subunit numbers (13) are indicated at *left*, and Complex III subunits are indicated at *right*. *C*, cross-linking of tRNA to native mitochondrial complexes. *Leishmania* and human mitochondrial complexes (Coomassie-stained profiles at *left* and *right*, respectively) were extracted with BAM buffer, and the extract was incubated with ^{32}P- and BU-labeled tRNA^{Ile} in the absence or presence of low specific activity tRNA^{Tyr} effector, UV-irradiated, and run on BN PAGE, before transfer to a nitrocellulose membrane and autoradiography. *D*, BU-labeled tRNA^{Ile} was incubated with purified RIC in the presence of tRNA^{Tyr} effector and/or anti-RIC8A antibody as indicated and then UV-irradiated, the complex dissociated and RNA-protein adducts resolved by urea-PAGE. *IP*, immunoprecipitate with anti-RIC8A antibody. *E*, effect of RIC8A knockdown on mitochondrial complexes. *Left panel*, the *L. tropica* RIC8A antisense transformant was induced with Tet, mitochondrial complexes extracted from 48-h induced (+) or uninduced (−) cultures were run on BN PAGE and Coomassie-stained. Bands labeled RIC and RIC′ were excised and run on SDS-PAGE and stained (*center panel*), or blotted and probed with anti-RIC8A antibody (*right panel*). *F*, interaction of tRNA with wild-type or RIC8A knockdown complex. BU-labeled tRNA^{Ile} (*left panel*) or tRNA^{Tyr} (*right panel*) was incubated with mitochondrial complexes from uninduced or tetracycline-induced RIC8A antisense transformants in the absence or presence of the indicated low specific activity effector, and the cross-linked complex was resolved by BN PAGE and visualized by autoradiography. The positions of the wild-type (*RIC*) and knockdown (*RIC′*) complex are indicated.

10-fold increase in the effective RIC8A concentration in the presence of the effector (Table 1), indicating enhanced exposure of the tRNA-binding site.

Effect of RIC8A/UCR6B Knockdown on Import in Leishmania—To assess the role of RIC8A/UCR6b *in vivo*, antisense RNA-mediated conditional knockdown (13) was carried out. The gene was cloned in the antisense orientation downstream of a Tet repressor-controlled T7 RNA polymerase promoter in the targeting vector pGET (Fig. 4*A*) and introduced into a *L. tropica* host expressing T7 RNA polymerase and the Tet repressor. Treatment of the transformant with tetracycline resulted in the rapid induction of anti-RIC8A/UCR6b RNA, with concomitant disappearance of the mRNA (Fig. 4*B*); other mRNAs, such as those for the mitochondrial Complex II iron-sulfur protein (Fig. 4*B*), were unaffected. The level of RIC8A/UCR6b protein was reduced to barely detectable levels within 48 h of induction (Fig. 4*B*), but there was no change in the overall polypeptide profile[3] or in the levels of other polypeptides such as the RIC subunit RIC1/F1α (Ref. 13 and Fig. 4*B*), attesting to the specificity of the targeting procedure. Moreover, the mitochondrial levels of proteins other than RIC8A/UCR6b were unaffected, and inner membrane proteins such as Complex II iron-sulfur protein remained resistant to trypsin in knockdown mitochondria until detergent disruption (Fig. 4*B*), indicating that the knockdown protocol did not affect the intactness of the organelle or the mitochondrial targeting of nucleus-encoded proteins.

In the absence of tetracycline, the anti-RIC8A/UCR6b transformants grew normally with a doubling time of ~24 h at 22 °C, which is close to the doubling time for cells transfected with the empty vector (Fig. 4*C*). The addition of tetracycline resulted in the cessation of growth from ~48 h (Fig. 4*C*); promastigotes became immotile, rounded or flask-shaped, and nonviable. Withdrawal of tetracycline after 72 h resulted in resumption of growth after a lag of 3–4 days, suggesting that the knockdown is reversible or that a few cells escape knockdown.

To check the effect of the knockdown on import, cytoplasmic and mitochondrial tRNAs of the induced cells were examined. There was an overall decline in the amount of mitochondrial tRNAs, although some tRNAs persisted (Fig. 4*D*). At the level of individual tRNA species, cytosolic levels were not appreciably altered, but mitochondrial tRNA^{Ile}(UAU), tRNA^{Val}(CAC), and tRNA$^{Met\text{-}e}$(CAU) (all type II tRNAs) were reduced to less than 10% of normal by 48 h (Fig. 4*E*). In contrast, there was an increase of 50–70% in the amount of mitochondrial tRNA^{Tyr}(GUA), tRNA^{Arg}(ACG), or tRNA^{Trp}(CCA) (all type I tRNAs) (Fig. 4*E*). This slight increase in signal intensity was maintained at different levels of input RNA in the RT-PCR assay (Fig. 4*F*). Thus, *in vivo* knockdown of RIC8A/UCR6b results in depletion of the mitochondrial type II tRNAs and a slight elevation of mitochondrial type I tRNAs.

In addition to the loss of mitochondrial tRNAs, severe pleiotropic effects on mitochondrial protein synthesis and the structure and function of respiratory complexes were observed upon knockdown of RIC8A/UCR6b. Although cytosolic protein synthesis was normal,[3] chloramphenicol-sensitive mitochondrial translation was reduced to barely detectable levels (Fig. 4*G*). The number of cells positive for cytochrome oxidase (or Complex IV) was reduced to ~16% of the uninduced value. There was simultaneously a reduction in the rate of O_2 uptake from 1.6 to 0.7 fmol min^{-1} cell^{-1} at 30 °C. The decline in mitochondrial tRNA import *in vivo* upon RIC8A/UCR6b knockdown could be due to this respiration defect, a defect in the import apparatus, or a combination of both factors. Although it is difficult to formally distinguish between these possibilities, it would be important to investigate whether the import function of RIC was affected by depletion of RIC8A/UCR6b.

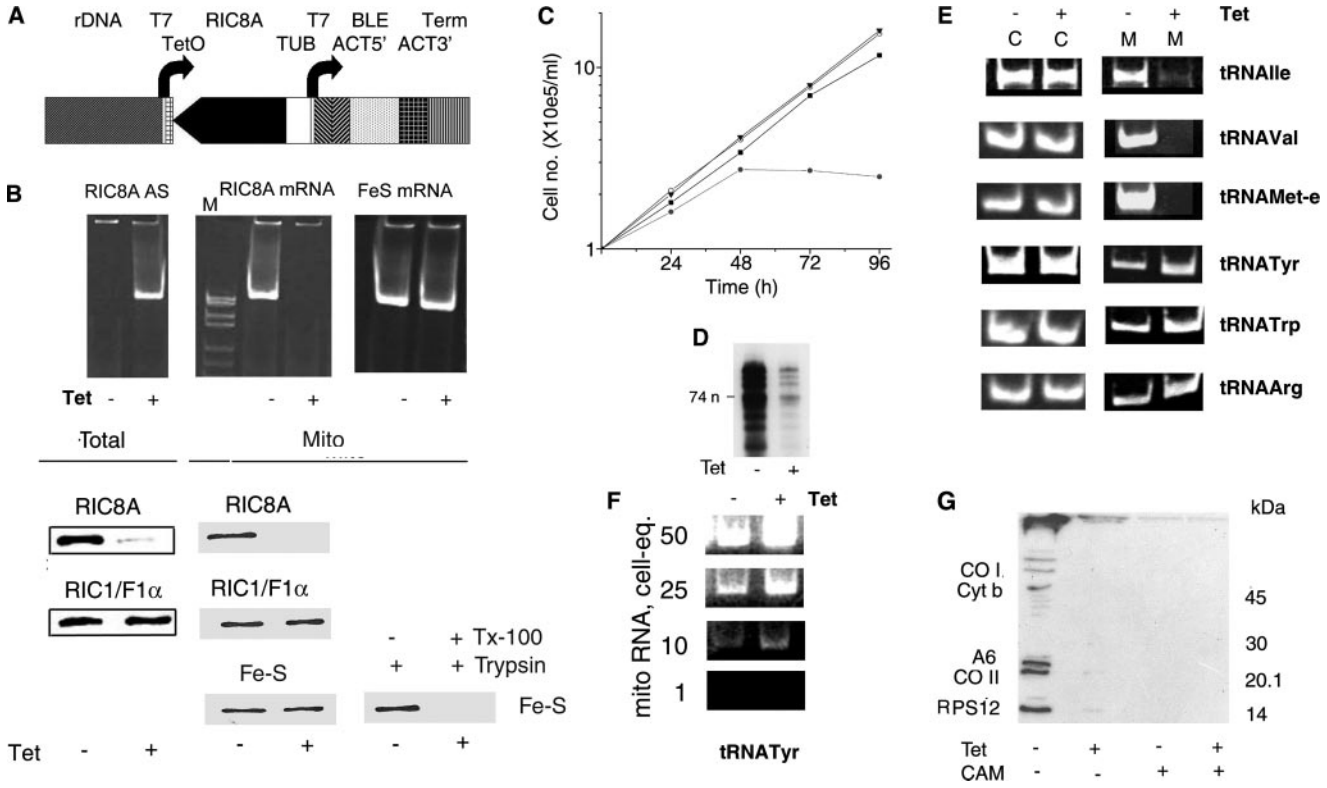

FIGURE 4. **Effects of RIC8A/UCR6b knockdown in *Leishmania*.** *A*, map of antisense vector pGET(AS)RIC8A. *rDNA*, ribosomal DNA spacer region; *T7*, T7 RNA polymerase promoter; *Tet O*, tetracycline operator; *TUB*, β tubulin; *ACT5′*, 5′ splice acceptor site of actin gene; *ACT3′*, 3′-untranslated region of actin; *BLE*, bleomycin resistance gene; *Term*, double T7 terminator. The *arrows* indicate transcription start sites. *B*, *upper panels*, RT-PCR analysis of RNA from pGET(AS)RIC8A transformed *L. tropica* 13–90 grown in the absence or presence of tetracycline for 48 h. *Left upper panel*, antisense RIC8A RNA; *middle upper panel*, RIC8A mRNA; *right upper panel*, Complex II iron-sulfur mRNA. *Lower panels*, immunodetection of RIC8A, RIC1/F1α and iron-sulfur protein in total (*left panel*) or mitochondrial (*center panel*) protein fractions. *Right panel*, the mitochondrial fraction was treated with trypsin in the absence or presence of Triton X-100, before immunoblotting with anti-iron-sulfur protein antibody. *C*, effect on cell growth at 22 °C. *Open circles*, pGET-transformed *L. tropica*, −tet; *filled triangles*, pGET-transformed, +tet; *open squares*, pGET(AS)RIC8A transformed, no tet; *filled circles*, pGET(AS)RIC8A transformed, +tet. *D*, end-labeled mitochondrial tRNA from uninduced or 48 h tet-induced pGET(AS)RIC8A transformed *L. tropica*. *E*, RT-PCR analyses of indicated tRNAs in the mitochondrial (*M*) or cytosolic (*C*) RNA fractions (10^2 cell-equivalent) of. uninduced or induced cells. *F*, RT-PCR of mitochondrial $tRNA^{Tyr}$ from induced or uninduced cells at different levels (as cell equivalent) of input RNA. *G*, mitochondrial translation. pGET(AS)RIC8A transformed *L. tropica* promastigotes, grown for 48 h in the absence or presence of tetracycline, were incubated with [^{35}S]methionine and cycloheximide in the absence or presence of chloramphenicol and labeled mitochondrial proteins resolved by SDS-PAGE Mitochondrially encoded proteins are indicated at *left*.

An Import-defective Knockdown Complex—When the respiratory complexes from knockdown cells were analyzed by BN PAGE, two large complexes, instead of the usual four, were observed (Fig. 3*E*). The larger complex corresponds to Complex V, as shown by two-dimensional analysis, except that the mitochondrially encoded A6 subunit was lacking, because of translational shut-off caused by defective tRNA import.[3] The smaller complex co-migrated with Complex IV but had the subunit profile of a subcomplex of RIC (RIC′; Fig. 3*E*). Western blotting showed that this subcomplex lacked RIC8A/UCR6b, as expected (Fig. 3*E*); the residual Coomassie-stained 21-kDa band is that of a different subunit (RIC8B). Additionally, RIC2, RIC3, and RIC7 were missing. These could be either mitochondrially encoded, and/or those that fail to be assembled in the absence of RIC8A/UCR6b. A lack of major mitochondrially encoded subunits also accounts for the failure to assemble Complexes III and IV.

The interaction of RIC′ with tRNAs was examined by photoaffinity cross-linking experiments. Although the wild-type complex was cross-linked to BU-labeled $tRNA^{Ile}$ (type II) in the presence of type I $tRNA^{Tyr}$ effector, the knockdown complex, lacking RIC8A/UCR6b, was not (Fig. 3*F*). Conversely, the wild-type complex interacted with BU-labeled $tRNA^{Tyr}$, but this type I interaction was inhibited by $tRNA^{Ile}$ (Fig. 3*F*), as previously observed with the affinity-purified complex (14). In contrast, RIC′ interacted with $tRNA^{Tyr}$ (Fig. 3*F*), but this interaction was not inhibited by type II $tRNA^{Ile}$ (Fig. 3*F*). Thus, knockdown of RIC8A/UCR6b resulted in the formation of a subcomplex in which both the binding of, and regulation by, type II tRNA were affected.

To determine whether the deficiency of RIC8A/UCR6b is sufficient to account for these defects, we tested mitochondrial extracts from normal or tetracycline-induced cells for functional reconstitution of import activity. When incorporated into liposomes, an extract from RIC8A knockdown cells was active in the import of type I, but not of type II tRNAs even in the presence of type I effector (Fig. 5*A*). Importantly, type II activity of the knockdown extract could be restored by adding back purified recombinant RIC8A/UCR6b and a type I tRNA to the reconstitution reaction (Fig. 5*B*); unrelated proteins such as bovine serum albumin were unable to substitute for RIC8A, and RIC8A/UCR6b alone was unable to protect the RNA from degradation by ribonuclease (Fig. 5*B*). Thus, the observed effects of RIC8A/UCR6b

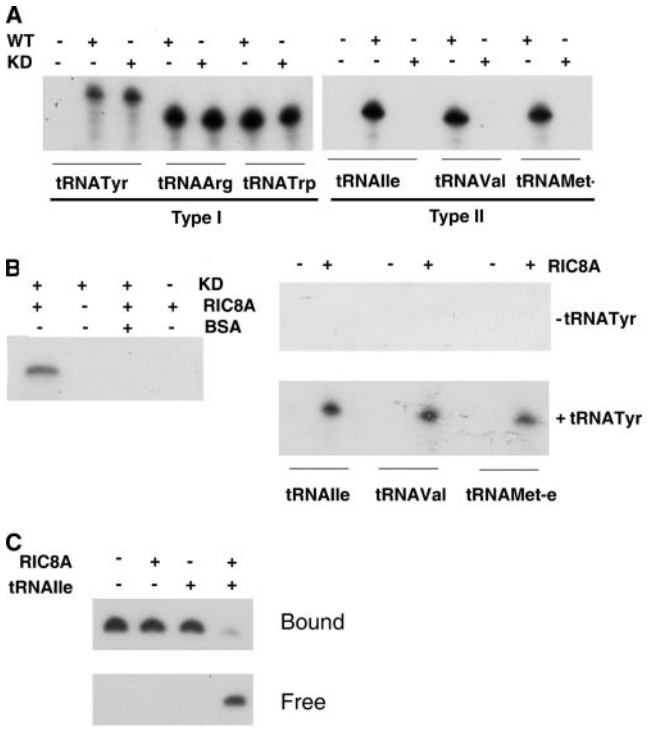

FIGURE 5. **Knockdown-induced defects in tRNA import.** *A*, import activity of mitochondrial extracts from uninduced (*WT*) or tetracycline-induced (*KD*) RIC8A antisense transformant cells. Liposomes were reconstituted with indicated extracts and assayed for uptake of the indicated tRNAs in presence of $tRNA^{Tyr}$ effector. *B*, import reconstitution. *Left panel*, reconstitution controls. Liposomes were incubated with knockdown mitochondrial extract, recombinant RIC8A and bovine serum albumin as indicated. *Right panels*, import of the indicated tRNAs into liposomes reconstituted with knockdown mitochondrial extracts in the absence or presence of purified RIC8A. Import assays were carried out in the absence (*upper panel*) or presence (*lower panel*) of low specific activity $tRNA^{Tyr}$ effector. *C*, regulation of binding of type I tRNA by RIC8A. ^{32}P-Labeled $tRNA^{Arg}$ was incubated in binding buffer with liposomes reconstituted with mitochondrial extract from RIC8A knockdown cells for 30 min at 4 °C, and the liposomes were washed and then reincubated for 1 h at 4 °C with low specific activity $tRNA^{Ile}$ effector and purified RIC8A, as indicated. The liposomes were centrifuged, and the RNA in the pellet (bound) and supernatant (free) was recovered.

knockdown were directly attributable to a role of this protein in type II tRNA import.

To examine the role of RIC8A/UCR6b in the regulation of type I tRNA, ^{32}P-labeled type I $tRNA^{Arg}$ was allowed to bind to proteoliposomes reconstituted with the RIC8A-deficient mitochondrial extract from knockdown cells. After a washing step to remove unbound $tRNA^{Arg}$, the liposomes were incubated with recombinant RIC8A/UCR6b in the absence or presence of type II $tRNA^{Ile}$, and the presence of free or liposome-bound $tRNA^{Arg}$ was monitored. Neither RIC8A/UCR6b nor $tRNA^{Ile}$, added individually, had any effect on the bound type I tRNA, but a combination of the two caused its dissociation from the liposomes and appearance in the supernatant (Fig. 5*C*). We conclude that the combination of a type II tRNA and RIC8A/UCR6b destabilizes the interaction between type I tRNA and its cognate receptor within the import complex. The role of RIC8A/UCR6b as a type II receptor was additionally confirmed by *in vitro* import experiments using antibody against the recombinant protein (supplemental Fig. S2).

DISCUSSION

In this report, we show that RIC8A/UCR6b, a 21-kDa subunit of the inner membrane RNA import complex, is required for the import of a specific subset of tRNAs (type II) into *Leishmania* mitochondria *in vivo*. The present results demonstrate the *in vivo* relevance of the ping-pong model of allosteric inter-tRNA interactions previously proposed on the basis of *in vitro* observations (14): 1) Knockdown of the protein in *Leishmania* specifically blocked uptake of type II tRNAs (Fig. 4). 2) Deficiency of RIC8A/UCR6b resulted in the formation of a partially defective import complex that was functional for the import of type I, but not of type II tRNAs, and was also defective in the regulation of type I tRNA (Figs. 3 and 5). 3) This defect could be rectified by recombinant RIC8A/UCR6b (Fig. 5). 4) RIC8A/UCR6b was specifically bound to type II tRNAs (Fig. 2). 5) Knockdown of the protein resulted in stimulation of import of type I tRNAs (Fig. 4). 6) RIC8A/UCR6b, in conjunction with type II tRNA, caused dissociation of type I tRNA from the complex (Fig. 5).

How does RIC8A/UCR6b recognize multiple type II tRNAs? We showed previously that the variable arm-T loop region of $tRNA^{Ile}$ contains a type II signal for transfer through the inner mitochondrial membrane (11, 12). Subsequently, the T stems of *T. brucei* $tRNA^{Ile}$ and $tRNA^{Met\text{-}e}$ (which are identical in sequence to the corresponding *Leishmania* species) have been found to contain import determinants (10). The T stems of all three type II tRNAs used in this study have the sequence YGRGU, of which the second G (paired to a C in the stem) is apparently critical (10); moreover, two major sequence classes of type II import aptamers selected by *in vitro* evolution contain the motif $UG_{3-4}U$ (11); finally, none of the type I tRNAs studied here contains this exact sequence in the T arm. Thus, it is conceivable this or a related motif directly interacts with RIC8A/UCR6b.

The intrinsic affinity of free RIC8A/UCR6b for type II tRNA is high (Table 1) and comparable with that of the type I receptor RIC1 for its cognate tRNA (13). However, within the native complex, in the absence of type I tRNA, this affinity is much lower, presumably because of the association of RIC8A with other subunits. Thus, the consequence of allosteric activation is to increase both the affinity as well as the number of binding sites exposed to tRNA (Table 1).

Knockdown of RIC8A/UCR6b led to a small but reproducible enhancement in the import of all three type I tRNAs (Fig. 4). The inhibitory effect of individual type II tRNAs, tested at a fixed concentration, is generally high but somewhat variable, *e.g.* $tRNA^{Ile}$ and $tRNA^{Val}$ are more effective than $tRNA^{Met\text{-}e}$ (supplemental Fig. S2), probably as a result of the different affinities of these tRNAs for RIC8A/UCR6b. Variable concentrations and affinities of the multitude of type II tRNAs encountered *in vivo* may account for the limited effect (50–70% increase) of RIC8A/UCR6b knockdown on import of type I tRNAs.

The RNA import complex is an assemblage of known and unknown proteins, some of which, such as RIC8A, RIC1 (13), and others[3] are also present in other respiratory complexes. The possibility of RIC being a nonspecific aggregate is ruled out

by the following observations. 1) It has a definite subunit composition that is reproducible in different preparations. 2) Knockdown of a particular subunit has a subunit-specific effect on this composition; for example, the subunit profile of the RIC8A knockdown complex (Fig. 3*E*) is different from that of the complex in RIC1 knockdown cells[3]; such effects can be reasonably explained by a defined assembly pathway but not by nonspecific aggregation. 3) Specific import-related interactions occur between different subunits of the complex, *e.g.* between RIC1 and RIC8A, and respond in a predictable way to knockdown of the interacting partners (Fig. 3*F*).

Subunit UCR6b of respiratory Complex III is absent from prokaryotes. The eukaryotic protein is highly conserved and has no enzymatic activity but is essential for cell viability, apparently because of its role in assembly of the functional complex (27). The *Leishmania* homologue, RIC8A/UCR6b, the product of a single gene, is shared between Complex III and the RNA import complex (Fig. 3), suggesting it to be a bi-functional protein. RIC8A/UCR6b (and probably, the corresponding protein from other kinetoplastid protozoa with almost identical sequence) has a tRNA-binding site that is exposed in RIC but not in Complex III (Fig. 3), *i.e.* the assembly of the protein in the two complexes is different. This suggests that one event in the evolution of import function may have been the acquisition of a tRNA binding N-terminal domain by *Leishmania* UCR6b as well as additional protein-protein interaction domains/motifs for alternate assembly. The emerging view of the RNA import complex is that of an assemblage of subunits including several bi-functional respiratory proteins. This differs from the yeast tRNA import system, which involves translation and mitochondrial protein import components (5, 6). Thus, different ancient metabolic pathways may have been the origin of the independent evolution of tRNA import in different species.

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REFERENCES

1. Bhattacharyya, S. N., and Adhya, S. (2004) *RNA Biol.* **1,** 84–88
2. Schneider, A., and Marechal-Drouard, L. (2000) *Trends Cell Biol.* **10,** 509–513
3. Simpson, A. M., Suyama, Y., Dewes, H., Campbell, D., and Simpson, L. (1989) *Nucleic Acids Res.* **17,** 5427–5445
4. Hancock, K., and Hajduk, S. L. (1990) *J. Biol. Chem.* **265,** 19203–19215
5. Tarassov, I., Entelis, N., and Martin, R. P. (1995) *J. Mol. Biol.* **245,** 315–323
6. Tarassov, I., Entelis, N., and Martin, R. P. (1995) *EMBO J.* **14,** 3461–3471
7. Nabholz, C. E., Horn, E. K., and Schneider, A. (1999) *Mol. Biol. Cell.* **10,** 2547–2557
8. Mahapatra, S., and Adhya, S. (1996) *J. Biol. Chem.* **271,** 20432–20437
9. Bhattacharyya, S. N., Mukherjee, S., and Adhya, S. (2000) *Mol. Cell. Biol.* **20,** 7410–7417
10. Esseiva, A. C., Marechal-Drouard, L., Cosset, A., and Schneider, A. (2004) *Mol. Biol. Cell* **15,** 2750–2757
11. Bhattacharyya, S. N., Chatterjee, S., and Adhya, S. (2002) *Mol. Cell Biol.* **22,** 4372–4382
12. Goswami, S., Chatterjee, S., Bhattacharyya, S. N., Basu, S., and Adhya, S. (2003) *Nucleic Acids Res.* **31,** 5552–5559
13. Goswami, S., Dhar, G., Mukherjee, S., Mahata, B., Chatterjee, S., Home, P., and Adhya, S. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 8354–8359
14. Bhattacharyya, S. N., Chatterjee, S., Goswami, S., Tripathi, G., Dey, S. N., and Adhya, S. (2003) *Mol. Cell Biol.* **23,** 5217–5224
15. Bhattacharyya, S. N., and Adhya, S. (2004) *J. Biol. Chem.* **279,** 11259–11263
16. Mahata, B., Bhattacharyya, S. N., Mukherjee, S., and Adhya, S. (2005) *J. Biol. Chem.* **280,** 5141–5144
17. Ghosh, A., Ghosh, T., Ghosh, S., Das, S., and Adhya, S. (1994) *Nucleic Acids Res.* **22,** 1663–1669
18. Ivens, A. C., Peacock, C. S., Worthey, E. A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M.-A., Adlem, E., Aert, R., Anupama, A., Apostolou, Z., Attipoe, P., Bason, N., Bauser, C., Beck, A., Beverley, S. M., Bianchettin, G., Borzym, K., Bothe, G., Bruschi, C. V., Collins, M., Cadag, E., Ciarloni, L., Clayton, C., Coulson, R. M. R., Cronin, A., Cruz, A. K., Davies, R. M., De Gaudenzi, J., Dobson, D. E., Duesterhoeft, A., Fazelina, G., Fosker, N., Frasch, A. C., Fraser, A., Fuchs, M., Gabel, C., Goble, A., Goffeau, A., Harris, D., Hertz-Fowler, C., Hilbert, H., Horn, D., Huang, Y., Klages, S., Knights, A., Kube, M., Larke, N., Litvin, L., Lord, A., Louie, T., Marra, M., Masuy, D., Matthews, K., Michaeli, S., Mottram, J. C., Müller-Auer, S., Munden, H., Nelson, S., Norbertczak, H., Oliver, K., O'Neil, S., Pentony, M., Pohl, T. M., Price, C., Purnelle, B., Quail, M. A., Rabbinowitsch, E., Reinhardt, R., Rieger, M., Rinta, J., Robben, J., Robertson, L., Ruiz, J. C., Rutter, S., Saunders, D., Schäfer, M., Schein, J., Schwartz, D. C., Seeger, K., Seyler, A., Sharp, S., Shin, H., Sivam, D., Squares, R., Squares, S., Tosato, V., Vogt, C., Volckaert, G., Wambutt, R., Warren, T., Wedler, H., Woodward, J., Zhou, S., Zimmermann, W., Smith, D. F., Blackwell, J. M., Stuart, K. D., Barrell, B., and Myler, P. J. (2005) *Science* **309,** 436–442
19. Bringaud, F., Peris, M., Zen K. H., and Simpson, L. (1995) *Mol. Biochem. Parasitol.* **71,** 65–79
20. Gao, X., Wen, X., Esser, L., Quinn, B., Yu, L., Yu, C. A., and Xia, D. (2003) *Biochemistry* **42,** 9067–9080
21. Schagger, H. (1996) *Methods Enzymol.* **264,** 555–566
22. Horvath, A., Berry, E. A., and Maslov, D. A. (2000) *Science* **287,** 1639–1640
23. Horvath, A., Kingan, T. G., and Maslov, D. A. (2000) *J. Biol. Chem.* **275,** 17160–17165
24. Speijer, D., Breek, C. K. D., Muijsers, A. O., Hartog, A. F., Berden, J. A., Albracht, S. P. J., Samyn, B., Van Beeumen, J., and Benne, R. (1997) *Mol. Biochem. Parasitol.* **85,** 171–186
25. Speijer, D., Muijsers, A. O., Dekker, H., de Haan, A., Breek, C. K. D., Albracht, S. P. J., and Benne, R. (1996)) *Mol. Biochem. Parasitol.* **79,** 47–59
26. Maslov, D. A., Nawathean, P., and Scheel, J. (1999) *Mol. Biochem. Parasitol.* **99,** 207–221
27. Schoppink, P. J., Berden, J. A., and Grivell, L. A. (1989) *Eur. J. Biochem.* **181,** 475–483