

A bifunctional tRNA import receptor from *Leishmania* mitochondria

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Edited by Dieter Söll, Yale University, New Haven, CT, and approved April 11, 2006 (received for review December 16, 2005)

In kinetoplastid protozoa, import of cytosolic tRNAs into mitochondria occurs through tRNAs interacting with membrane-bound proteins, the identities of which are unknown. The inner membrane RNA import complex of *Leishmania tropica* contains multiple proteins and is active for import *in vitro*. RIC1, the largest subunit of this complex, is structurally homologous to the conserved α subunit of F1 ATP synthase. The RIC1 gene complemented an *atpA* mutation in *Escherichia coli*. Antisense-mediated knockdown of RIC1/F1 α in *Leishmania* resulted in depletion of several mitochondrial tRNAs belonging to distinct subsets (types I and II) that interact cooperatively or antagonistically within the import complex. The knockdown-induced defect in import of type I tRNAs was rectified in a reconstituted system by purified RIC1/F1 α alone, but recovery of type II tRNA import additionally required a type I tRNA. RIC1/F1 α formed stable complexes with type I, but not type II, tRNAs through the cooperation of its nucleotide binding and C-terminal domains. Thus, RIC1/F1 α is a type I tRNA import receptor. As expected of a bifunctional protein, RIC1/F1 α is shared by both the import complex and by respiratory complex V. Alternative use of ancient respiratory proteins may have been an important step in the evolution of tRNA import.

ATP synthase α subunit | import factor

The highly derived mitochondrial genomes of protozoa of the order Kinetoplastida, including *Leishmania* and *Trypanosoma* species, contain ≈ 18 protein-coding genes, and two rRNA genes, but completely lack tRNA genes (1). Thus, mitochondrial translation depends on import of large numbers of nucleus-encoded tRNAs from the cytoplasm (2, 3). Mitochondrial tRNA import also occurs in many other species, including higher plants, yeast, and marsupials (reviewed in refs. 4 and 5).

In yeast, import of a single tRNA^{Lys} requires cytosolic carriers and apparently occurs through protein import channels (6, 7). In contrast, kinetoplastids employ a membrane-bound system that recognizes specific import signal motifs on tRNAs, and soluble carriers are not essential (8, 9). Moreover, importable RNAs interact cooperatively and antagonistically with one another at the inner membrane of *Leishmania* mitochondria *in vitro* and in transiently transfected cells, leading to their classification into type I RNAs, which are directly transported through the inner membrane, and type II RNAs, which require the presence of type I RNAs; conversely, type II RNAs inhibit the inner membrane transfer of type I RNAs (10, 11).

A large, functional multiprotein import complex (the RNA import complex, or RIC) was isolated from the inner membrane of *Leishmania* mitochondria (12, 13). At least two tRNA-binding proteins are associated with this complex, having the properties of type I and type II receptors, respectively (12). However, the identities of these proteins, and their relevance to allosteric regulation of import *in vivo*, remain undefined. To begin to address these issues, we report the identification of one of the subunits of RIC that binds type I tRNAs and is essential for the import of type I as well as type II tRNAs *in vivo*.

Results

The Sequence of RIC1. RIC isolated by affinity chromatography consistently resolves into nine bands (RIC1–9) ranging in mo-

lecular mass from 62 to 19 kDa, in the relative proportion of 1:2:1:1:1:3:1:3:3 (see Fig. 5B). The present subunit numbering system for RIC replaces the previously used system based on apparent molecular mass deduced from electrophoretic mobility (12). RIC45p and RIC21p are henceforth designated as RIC1 and RIC8A, respectively. RIC1, which migrates abnormally in some gel systems, is now known from the gene sequence (see below) to be a 62-kDa protein. Three minor (substoichiometric) bands of 26, 28, and 30 kDa, which are detected in the affinity-purified complex only by silver staining (12), are ignored as possible contaminants.

Mass spectrometric sequencing of RIC1 revealed the presence of a protein encoded by an ORF of 574 aa residues, annotated in the *Leishmania major* database (14) as the α -subunit of the mitochondrial F1F0 ATP synthase (designated herein as F1 α ; Fig. 1A). The cloned *Leishmania tropica* gene (coding region) was independently sequenced and found to be identical to that from *L. major*. Mitochondrial or bacterial F1 α is a conserved protein containing three domains: an N-terminal domain composed of a β barrel, a central ATP binding domain, and a C-terminal domain comprised of an α -helical bundle (15). Sequence alignment (Fig. 6, which is published as supporting information on the PNAS web site) shows an overall 50% identity of *Leishmania* RIC1 with the bovine F1 α , with most of the identities located in the nucleotide-binding domain. Homology modeling reinforced the similarity in secondary structure between RIC1 and F1 α (Fig. 1B), particularly in the central domain (RIC1 residues 117–406).

However, significant differences from the canonical F1 α were noted: (i) the N-terminal domain of RIC1 (residues 27–116) is predicted to have a less extensive β barrel than the bovine protein, and (ii) similarity in the C-terminal domain extends between residues 407 and 501. The *Leishmania* protein contains an extension at the C terminus (Fig. 1A) that is predicted to form three α helices.

OX-PHOS Function of RIC1. The structure-based prediction of the gene as a subunit of F1 ATP synthase was tested by its ability to complement a mutation in the F1 α gene and restore respiratory function. The RIC1 gene was PCR-amplified from *Leishmania* genomic DNA and cloned, and fragments were expressed in *Escherichia coli* harboring the *atpA401* mutation in the F1 α gene; such a mutation renders the bacteria unable to grow on nonfermentable carbon sources such as succinate (16). RIC1[39–574], containing most of the N-terminal domain and the remainder of the gene, complemented the mutation, resulting in growth on succinate, but RIC1[109–574], lacking the N-terminal domain, did not (Fig. 1C). Thus, despite structural differences with the canonical F1 α , *Leishmania* RIC1 functions in ATP synthesis in *E. coli*. The N-terminal β -barrel domain makes essential

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: BN, blue native.

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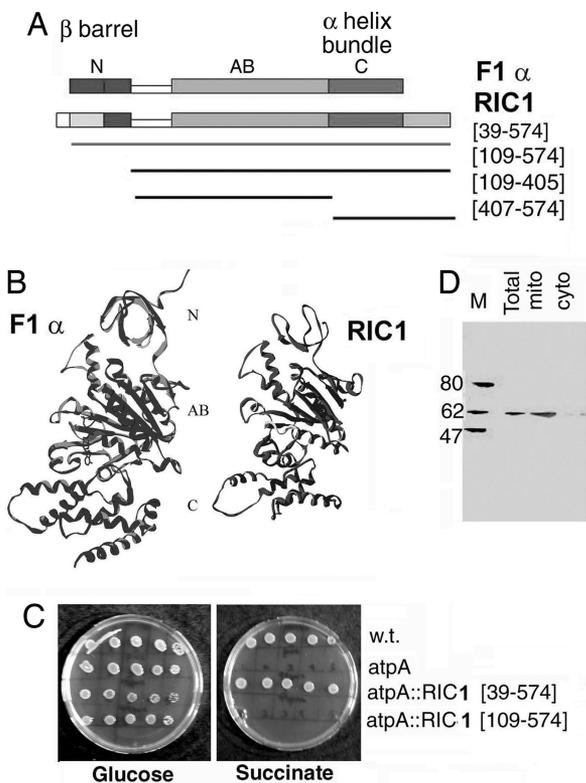


Fig. 1. Structural and functional homology of RIC1 with ATP synthase subunit α . (A) RIC1 and AtpA conserved domain (COG0056) of F1 ATP synthase subunit α , showing N-terminal (N), ATP binding (AB), and C-terminal (C) domains. (B) Homology model of RIC1 secondary structure backbone compared to that of bovine F1 ATPase α subunit. (C) Complementation of *E. coli* AtpA mutation by *Leishmania* RIC1. Serial dilutions (from 10^5 to 10 cells, left to right) of strain AN120 (AtpA), strain AN180 (the isogenic wild-type) or AN120 transfected with RIC1[39–574] or RIC1[109–574] were spotted on minimal media containing either glucose or succinate as carbon source. (D) Immunoblot of protein (100 μ g) of total, mitochondrial (mito), or cytosolic (cyto) fractions of *L. tropica* promastigotes using anti-RIC1 serum as probe.

contacts with the β subunit of F1 ATP synthase, as shown by structural and mutational studies (15, 17, 18); the relevant interaction motifs have probably been conserved in the protozoal protein, allowing assembly of a functional complex V.

The *Leishmania* genome contains two tandemly repeated identical copies of the F1 α gene on chromosome 5 (systematic gene names Lmj05.0500 and Lmj05.0510), and no other homologues have been detected (14). Moreover, only a single 62-kDa protein is located in the mitochondria of *Leishmania* promastigotes (Fig. 1D). These observations lead to the hypothesis that RIC1, after being targeted into the mitochondrial compartment by its putative import signal (Fig. 6), performs the dual functions of tRNA import and ATP synthesis.

Effect of RIC1 Knockdown on tRNA Import in *Leishmania*. To assess the role of RIC1 in mitochondrial tRNA import in *Leishmania*, a conditional antisense knockdown strategy was adopted. The gene was cloned in the antisense orientation into the vector pGET (Fig. 7, which is published as supporting information on the PNAS web site), downstream of a T7 polymerase promoter regulated by the Tet repressor. The recombinant (Fig. 2A) was integrated into the ribosomal RNA spacer region of an *L. tropica* host (strain 13–90) expressing the genes for the polymerase and repressor.

In presence of tetracycline, anti-RIC1 RNA was induced, with concomitant down-regulation of the corresponding mRNA (Fig.

2B). Control mRNAs, such as that for RIC8A, another RIC subunit, were not affected (Fig. 2B). Although there was no change in the total protein profile, RIC1 protein became nearly undetectable (Fig. 2C). The levels of other mitochondrial proteins such as the F1 ATP synthase β subunit or complex II Fe-S protein were unaffected; moreover, inner membrane markers remained inaccessible to protease until released by detergent treatment of the mitochondrial fraction (Fig. 2C). Thus, knockdown of RIC1 had no appreciable effect on targeting of proteins into, or the intactness of, the mitochondria. Growth of the antisense transformants ceased between 24 and 36 h (Fig. 2D); promastigotes became rounded and nonviable. Growth of control cells transformed with the vector alone was not affected by tetracycline.

RIC1 knockdown had a severe effect on the levels of mitochondrial tRNAs. The overall amount of mitochondrial tRNA declined (Fig. 2E). There was no significant change in cytosolic tRNAs (Fig. 2F). In contrast, the levels of several nucleus-encoded tRNAs in the mitochondria were reduced to <10% of normal (Fig. 2F).

To determine whether a deficiency of other respiratory components not associated with tRNA import has a similar effect on mitochondrial tRNA content, knockdown of the β subunit of F1 ATP synthase (which does not copurify with RIC; see below), was effected (details in Fig. 8, which is published as supporting information on the PNAS web site); this resulted in lethality, but there was no appreciable effect on the content of mitochondrial tRNAs (Fig. 2F). Thus, down-regulation of RIC1/F1 α has a specific effect, not related to its respiratory function, on the levels of cytosolic tRNAs in mitochondria *in vivo*.

Reduction of tRNA levels caused downstream defects in mitochondrial function. Although there was no alteration in total (mainly cytosolic) protein synthesis upon tetracycline induction, cycloheximide-resistant, chloramphenicol-sensitive translation in mitochondrial fractions was abolished (Fig. 2G). The activity of cytochrome oxidase (complex IV) became nearly undetectable, whereas the rate of O₂ consumption of RIC1 knockdown *Leishmania* was reduced to \approx 20% of normal. This reduction is expected, because the major complex IV subunits (COI, COII, and COIII) are encoded in the mitochondrial genome (1). In contrast, knockdown of F1 β had no effect on COX activity (data not shown), and mitochondrial translation was nearly normal (Fig. 8). Because translation depends on the nucleoside triphosphate (ATP, GTP) pool, the persistence of protein synthesis implies that, at the time of harvesting of the knockdown cells, intracellular ATP pools had not declined to subcritical levels. Thus, ATP depletion is unlikely to be the cause of the reduction in mitochondrial tRNA import observed upon knockdown of RIC1.

Among the tRNAs whose mitochondrial levels depend on RIC1 *in vivo* (Fig. 2F), tRNA^{Tyr} and tRNA^{Ile} belong to the type I and II categories, respectively, as defined by *in vitro* RIC-reconstituted liposome assays (12). We first checked the status of the other four tRNAs by monitoring their uptake into RIC-liposomes in the absence or presence of low concentrations of tRNA^{Tyr} or tRNA^{Ile} as standard effector. Uptake of tRNA^{Arg} and tRNA^{Trp} did not require the presence of type I effector, and was inhibited by type II effector. Conversely, uptake of tRNA^{Val} and tRNA^{Met-c} depended on type I tRNA (Fig. 3A). Thus, tRNA^{Tyr}, tRNA^{Arg} and tRNA^{Trp} have the properties of type I, whereas tRNA^{Ile}, tRNA^{Val}, and tRNA^{Met-c} belong to the type II category. Similar results were obtained by using other combinations of the two types of tRNA (data not shown).

To determine whether the reduced levels of mitochondrial tRNAs in RIC1-knockdown cells were caused by a defect in the import apparatus, liposomes were reconstituted with mitochondrial extracts from knockdown cells and assayed for import. Extracts from uninduced cells (WT) directed import of all of the

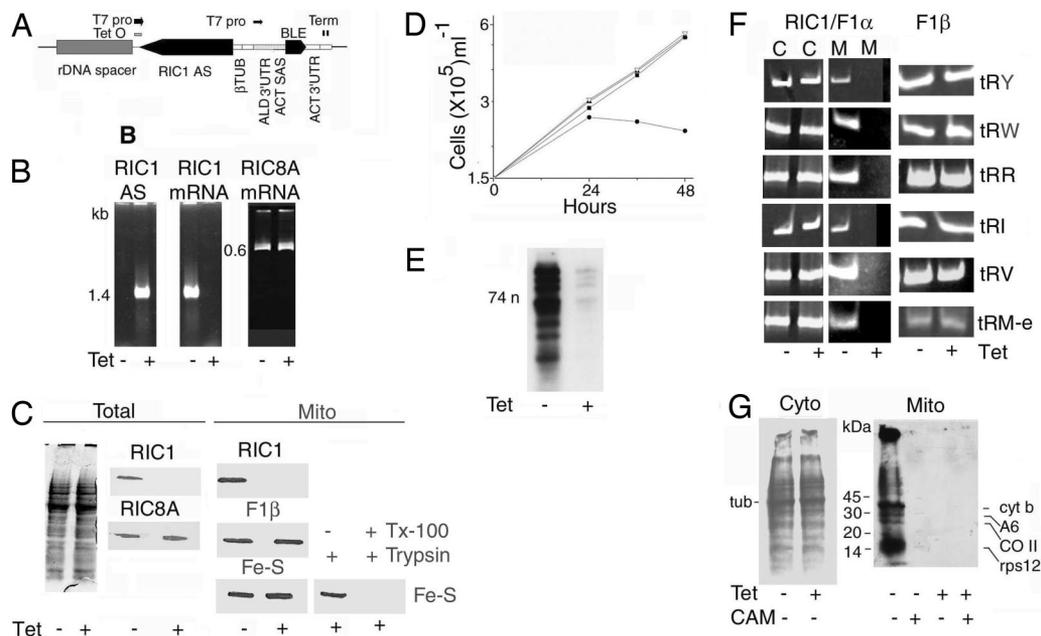


Fig. 2. Antisense-mediated knockdown of RIC1/F1 α . (A) Schematic diagram of the expression cassette of knockdown vector pGET(AS)RIC1/F1 α . rDNA, ribosomal DNA spacer region; T7 pro, T7 RNA polymerase promoter; Tet O, tetracycline operator; RIC1/F1 α AS, RIC1[109–574] in antisense orientation; TUB, β tubulin; ALD, aldehyde dehydrogenase; ACT SAS and ACT 3' UTR, 5' splice acceptor site and 3' UTR, respectively, of actin gene; BLE, bleomycin resistance gene; Term, double T7 terminator. Arrows indicate transcription start sites. (B) RT-PCR assay of RIC1/F1 α antisense RNA (Left), RIC1/F1 α mRNA (Center), or RIC8A mRNA (Right) in pGET(AS)RIC1-transformed *L. tropica* 13–90 (10^5 cell equivalent) grown for 24 h in absence (–) or presence (+) of tetracycline. (C) (Left) Total Coomassie-stained protein from 10^7 cells and immunoblot with anti-RIC1/F1 α (Upper) or anti-RIC8A (Lower). (Right) Mitochondrial protein blots probed with antibodies against RIC1/F1 α , F1 β subunit, or complex II Fe-S protein. At the far right, mitochondrial fractions were treated with trypsin (20 μ g/ml, 20 min, 4°C) in absence or presence of 0.5% Triton X-100. (D) Growth curves at 22°C. Open triangles, pGET-transformed *L. tropica*, –tet; filled triangles, pGET-transformed, +tet; squares, pGET(AS)RIC1-transformed, –tet; circles, pGET(AS)RIC1-transformed, +tet. (E) Total end-labeled mitochondrial tRNA in normal or RIC1-knockdown cells. (F) RT-PCR assay of tRNA^{Tyr} (tRY), tRNA^{Trp} (tRW), tRNA^{Arg} (tRR), tRNA^{Ile} (tRI), tRNA^{Val} (tRV), and tRNA^{Met-e} (tRM-e) in cytosolic (C) or mitochondrial (M) RNA from 10^3 RIC1/F1 α (Left) or F1 ATPase β subunit (Right) knockdown cells. (G) [³⁵S]Methionine-labeled proteins from uninduced or tet-induced pGET(AS)RIC1-transformed cells cultured in absence (Left) or presence (Right) of cycloheximide. Chloramphenicol was additionally present in the cycloheximide-treated cultures as indicated, and the mitochondrial fractions were isolated (Right).

tRNAs tested into liposomes, but tetracycline-induced cell extracts (KD) were completely inactive (Fig. 3 B and C). Import activity was restored under the following conditions. For import of type I tRNAs, addition of recombinant RIC1 (109–574) was

sufficient, and the type I tRNA^{Tyr} had no effect. On the other hand, all of the type II tRNAs required RIC1 as well as type I effector (Fig. 3C). In control reactions, unrelated proteins such as BSA could not replace RIC1, and RIC1 alone was unable to induce import (Fig. 3B).

Thus, deficiency of RIC1 affects import of multiple type I as well as type II tRNAs *in vivo*. Moreover, because the complementing fragment (containing residues 109–574; Fig. 1) lacks the N-terminal domain, this domain is dispensable for import activity. Because of its functions as a respiratory component and as an import factor, the protein is henceforth designated RIC1/F1 α .

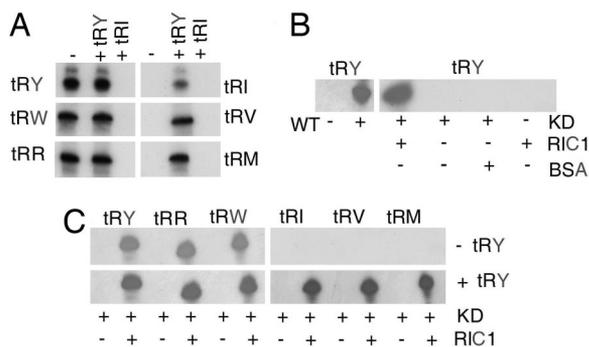


Fig. 3. Requirement of RIC1/F1 α for import of type I and II tRNAs. (A) Regulation of import by type I and II effector tRNAs. High specific activity import substrates (5 nM) were incubated in the absence or presence of low specific activity tRNA^{Tyr} or tRNA^{Ile} effector (0.5 nM) and assayed for import into RIC-reconstituted proteoliposomes. (B) Import reconstitution assay. Mitochondrial extract from uninduced (WT) or 24-h tet-induced (KD) pGET(AS)RIC1-transformed cells was incubated with or without recombinant RIC1/F1 α or with BSA (BSA), in presence of liposomes, which were then assayed for import of tRNA^{Tyr}. (C) Import of the indicated tRNAs (5 nM) in absence or presence of low specific activity tRNA^{Tyr} effector (0.5 nM), into liposomes reconstituted with knockdown extracts in the absence or presence of recombinant RIC1/F1 α .

tRNA-Binding Activity of RIC1/F1 α . Bacterially expressed recombinant RIC1/F1 α was incubated with radiolabeled tRNAs and complex formation monitored by the electrophoretic mobility shift assay (EMSA). Of the six tRNAs tested, three, tRNA^{Tyr}, tRNA^{Trp} and tRNA^{Arg} (all type I tRNAs), formed specific complexes, whereas the remaining three (all type II tRNAs) did not (Fig. 4A). Thus, RIC1/F1 α is a type I-specific tRNA binding protein. From RNA and protein titration experiments (Fig. 4B), the dissociation constant (K_d) of the tRNA^{Tyr}:RIC1/F1 α complex was estimated to be 0.55 nM at 4°C.

To map the tRNA binding region within the protein, different fragments of RIC1/F1 α were incubated with radiolabeled tRNA^{Tyr}. A fragment covering the central and C-terminal domains (residues 109–574; Fig. 1A) had tRNA-binding activity. In contrast, neither the central domain (residues 109–405) nor the C-terminal domain (residues 406–574) formed a complex. Binding was restored in presence of equimolar amounts of the

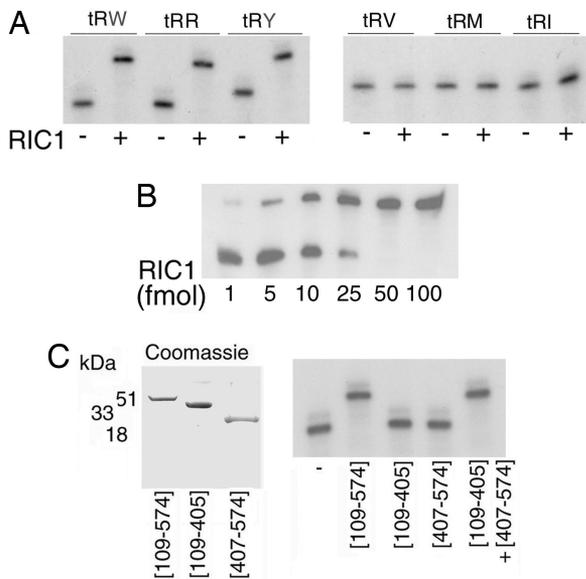


Fig. 4. Binding of tRNAs to RIC1/F1 α . (A) EMSA of 32 P-labeled tRNAs (10 fmol) incubated with or without recombinant RIC1/F1 α (50 fmol) in 5 μ l. (B) Titration of tRNA^{Tyr} (10 fmol) with indicated amounts of RIC1/F1 α (10- μ l reactions). (C) (Left) Truncated versions of the RIC1/F1 α gene expressed in *E. coli* stained with Coomassie blue. (Right) EMSA of 32 P-labeled tRNA^{Tyr} incubated with subfragments of RIC1/F1 α alone or in combination (conditions as in A).

two domains (Fig. 4C), indicating that both domains are required for the interaction.

Presence of RIC1/F1 α in Two Mitochondrial Complexes. We examined the mitochondrial inner membrane respiratory complexes of *L. tropica* resolved by Blue Native gel electrophoresis (BN PAGE). As shown previously for *L. tarentolae* (19, 20), *Crithidia* (21, 22), and the plant kinetoplastid parasite *Phytomonas* (23), four bands were observed. Three of these correspond to complexes III (ubiquinol cytochrome *c* reductase), IV (cytochrome oxidase), and V (F1F0 ATPase), respectively (Fig. 5A). The composition of the largest complex, found only in kinetoplastid protozoa, was analyzed by SDS/PAGE in the second dimension. This showed that (i) its polypeptide profile is identical to that of the functional RNA import complex independently isolated from mitochondrial inner membranes by affinity chromatography (Fig. 5B), and (ii) it is different from the other respiratory complexes, particularly complex V (Fig. 5D). For example, the F1 synthase β subunit is present exclusively in complex V (Fig. 5C and D).

Western blot analysis of the complexes using anti-RIC1/F1 α antibody gave two bands corresponding to complex V and RIC (Fig. 5C). Resolution of the individual complexes in the second dimension by SDS/PAGE, followed by Western blot analysis, confirmed that a similar or identical protein is present in both (Fig. 5D). Moreover, of the subunits of RIC and *Leishmania* complex V resolved by SDS/PAGE, only RIC1/F1 α interacts on Northwestern blots with tRNA^{Tyr} (Fig. 5D). Thus, RIC1/F1 α is shared by the import complex and complex V.

When a Western blot of the denatured complexes was probed with radiolabeled tRNA^{Tyr}, complex V as well as RIC became labeled, as expected by the presence of the same tRNA binding component, RIC1, in both complexes (Fig. 5E). In contrast, the human complex V, although containing F1 α , did not have any tRNA-binding activity (Fig. 5E), illustrating the intrinsic differences between the mammalian and the protozoal homologues.

Because *Leishmania* RIC1/F1 α in both RIC and complex V is capable of binding tRNA (Fig. 5E), we examined whether the

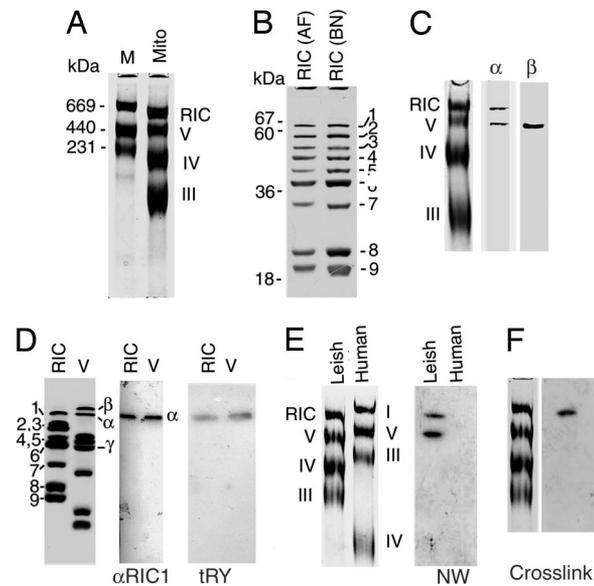


Fig. 5. Relationship between the RNA import complex and respiratory complexes of *Leishmania*. (A) *L. tropica* mitochondrial protein (200 μ g) run in 6% BN PAGE along with molecular weight markers (M). (B) The largest BN PAGE band was excised from BN gel, denatured and run on SDS/12% PAGE along with affinity purified RIC (AF). RIC band numbers are indicated at right. (C) Immunoblot of mitochondrial complexes probed with anti-RIC1/F1 α or F1 β antibody. (D) Second-dimension analysis of RIC and complex V, showing Coomassie-stained subunits (Left), anti-RIC1/F1 α immunoblot (Center), and Northwestern blot probed with 32 P-labeled tRNA^{Tyr} (Right). (E) (Left) *Leishmania* and human mitochondrial complexes resolved by BN PAGE. (Right) Northwestern blot probed with 32 P-labeled tRNA^{Tyr}. (F) Dodecyl maltoside extracted mitochondrial complexes were incubated with 32 P- and BrdU-labeled tRNA^{Tyr} followed by UV-cross-linking, BN PAGE, transfer to nitrocellulose membrane, and autoradiography. Coomassie-stained complexes are shown at left.

tRNA-binding site of the protein, assembled in the two native complexes, is equally accessible to tRNA. The mixture of native complexes obtained by extraction of *Leishmania* mitochondria was incubated with tRNA^{Tyr} doubly labeled with 32 P- and 5-bromouridine, a photoactivable nucleoside analogue, and after UV treatment, crosslinked ribonucleoprotein complexes were resolved by BN PAGE. Only RIC, and not complex V, was tagged with tRNA (Fig. 5F). This finding indicates that RIC1/F1 α is assembled differently in the two complexes, with the tRNA-binding site being exposed in the former but not in the latter.

Discussion

We present evidence that *Leishmania* RIC1/F1 α is a type I tRNA import receptor. It specifically binds to several type I, but not type II, tRNAs (Fig. 4). Moreover, a deficiency of RIC1/F1 α *in vivo* affects the import of both types of tRNAs (Fig. 2), as predicted by the “ping-pong” model of inter-tRNA interactions (12). We have recently identified a second RIC subunit with the properties of a type II receptor (unpublished data).

The import signal of the type I tRNA^{Tyr} is located within the D arm (24, 25). This region contains the sequence AGAGY that is also present in the D arms of tRNA^{Trp} and tRNA^{Arg}, as well as in the major sequence class of type I import aptamers obtained by *in vitro* evolution (10), and could form the core recognition motif for RIC1/F1 α . In contrast, none of the type II tRNAs tested contain this motif. Binding of tRNA requires both the nucleotide binding and the C-terminal domains of RIC1/F1 α (Fig. 4), implying that either the tRNA binding site spans both domains, or that binding to one domain requires conformational

activation by the other. The presence of the C-terminal extension in the kinetoplast protein, absent from the ubiquitous F1 α structure (Fig. 1), correlates with tRNA-binding activity of the *Leishmania*, but not the mammalian, protein (Fig. 5).

RIC1/F1 α is identical to the α subunit of *Leishmania* F1 ATP synthase, and is capable of functioning both in oxidative phosphorylation and as an import factor. RIC1/F1 α is the product of a single (duplicated) gene, with no isoforms being detected at the level of mRNA or protein, and is present in RIC as well as in complex V, but its disposition in the two complexes is apparently different, with the RNA-binding site exposed in the former but not in the latter (Fig. 5). Moreover, the N-terminal β -barrel domain of RIC1/F1 α is required for ATP synthetic activity (Fig. 1), but not for import function (Figs. 3 and 4), reflecting its association with the F1 β subunit in complex V, but not in RIC. Thus, although the two functions of RIC1/F1 α both impact mitochondrial metabolism, they are distinct in terms of their structural requirements.

Mitochondrial tRNA import occurs in a wide range of species across the phylogenetic scale, but it appears to have evolved independently in different lineages. The occurrence of a bifunctional tRNA-binding F1 α suggests that ancient respiratory components provided the raw material for the evolution of this function. Additional domains (such as the extra C-terminal tail for RIC1/F1 α) imparting new capabilities (tRNA binding) may have been incorporated into conserved proteins without disruption of their original function. Appearance of new interaction patches on the molecule would then create the possibility of alternate associations with different sets of proteins to form functionally distinct complexes.

Materials and Methods

Sequence Analysis and Homology Modeling. RNA import complex was purified from *L. tropica* inner mitochondrial membranes as described (12), and resolved by SDS/PAGE. Tryptic peptides of RIC1 were subjected to LC/MS and MS/MS analysis at the W. M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia, Charlottesville). Sequenced peptides (Table 1, which is published as supporting information on the PNAS web site) were matched against the *L. major* database (www.genedb.org/genedb/leish/index.jsp) to identify the corresponding gene by using BLAST or SEQUEST program. The RIC1 sequence was aligned with the AtpA domain (COG0056) of the Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) using CLUSTALW. Secondary structure predictions were made by the Predict-Protein server (www.cubic.bioc.columbia.edu/predictprotein). Automated homology modeling of the *Leishmania* protein was performed by SWISS-MODEL (www.expasy.ch/swissmod/SWISS-MODEL.html), using the crystallographic structure of bovine F1 ATPase subunit α as template.

Gene Cloning. Sense and antisense primers corresponding to various regions of the coding sequence (Table 2, which is published as supporting information on the PNAS web site) were used to amplify intact or truncated versions of the RIC1/F1 α gene from *L. tropica* strain UR6 genomic DNA. Details of cloning procedures are in *Supporting Text*, which is published as supporting information on the PNAS web site.

Genetic Complementation. *E. coli* strains AN120 (*argE3, thi-1, str^R, unc401/atpA401*) and the isogenic AN180 (*argE3, thi-1, str^R*) (16) were obtained from the Coli Genetic Stock Center (Yale University, New Haven, CT). Strain AN120 was transfected with recombinant pUC19 containing the appropriate RIC1/F1 α fragment and transformants grown on SOB agar (26) containing 100 μ g/ml ampicillin. To test for complementation, cells were spotted on medium 56 minimal agar (16) containing thiamine

(0.2 μ M), arginine (0.8 mM), 0.5 mM IPTG, and either glucose or succinate (30 mM each) as the carbon source.

Conditional Knockdown. Details of construction of the host strain and the inducible antisense expression vector are provided in *Supporting Text*. *L. tropica* 13-90 was transfected with pGET (AS)RIC1/F1 α and transformants selected on semisolid agar containing G418, hygromycin and 2.5 μ g/ml bleomycin. Clones were grown in Medium 199 containing the same antibiotics. Cultures were induced with 1 μ g/ml tetracycline, and cell growth was monitored. To minimize nonspecific, pleiotropic effects such as ATP depletion that are inevitable upon cell death, intracellular parameters were measured at the time point (24 h) 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 (25). The crude mitochondria were treated with DNase and RNase before RNA isolation. Reverse transcription of RNA from 10² to 10⁵ cells was performed with Superscript II reverse transcriptase (Invitrogen) and the appropriate primer, as follows: (i) for antisense RNA, the sense primer corresponding to the 5' end of the coding region of the corresponding gene; (ii) for mRNA or tRNA, the antisense primer complementary to the 3' end of the gene (Table 2). To prevent interference by double-stranded RNA in antisense expressing cells, the RNA was heat-denatured at 95°C and quick-chilled before the primer annealing step. The cDNA was then amplified with TaqDNA polymerase after addition of the second primer. Quantitative estimates were obtained by varying the input RNA to ensure proportionality with the PCR signals.

Preparation of Recombinant RIC1/F1 α . RIC1/F1 α fragments were expressed in *E. coli* as glutathione *S*-transferase (GST) fusion proteins and digested with thrombin, as described in *Supporting Text*. Thrombin digests were run on SDS/PAGE to separate the recombinant protein from the GST. After KCl staining (27), the band of pure recombinant protein was excised, and the protein was eluted with buffer PEB (0.05 M Tris-HCl, pH 8.0/0.2 M NaCl/0.1 mM EDTA/5 mM DTT/0.2% SDS/0.1 mg/ml BSA) overnight at 37°C and stored as such at -70°C. For RNA binding assays, the recombinant protein was refolded by diluting 20-fold into TETN250 buffer (12) containing 0.1% Triton X-100, and incubated for 2 h at 4°C. Polyclonal antibody against gel-purified recombinant RIC1[109-574] was raised in BALB/c mice.

BN PAGE. Respiratory complexes were solubilized as per Schagger's protocol (28) from purified mitochondria of *L. tropica* or the human HepG2 cell line (200 μ g of protein), or the crude mitochondrial fraction (prepared as in ref. 25) from 10⁷ cells, 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. The extract was clarified by centrifugation and concentrated to \approx 10 μ l per 200 μ g 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% BN gels as described (28). Bands were visualized by Coomassie staining. For two-dimensional analysis, gel slices of complexes resolved by BN PAGE were incubated in denaturing buffer (0.125 M Tris-HCl, pH 6.8/1% SDS/1% 2-mercaptoethanol) for 40 min at 37°C, then loaded on a 10 or 12% denaturing polyacrylamide gel for SDS/PAGE.

Preparation of Radiolabeled tRNA. Sequences of tRNA^{Arg}₁(ACG), tRNA^{Trp}(CCA), tRNA^{Val}(CAC), and tRNA^{Met-e}(CAU) were obtained from the *L. major* database, and the corresponding genes were amplified from *L. tropica* genomic DNA using 5' and 3' primers (Table 2) and cloned downstream of the T7 poly-

merase promoter in vector pGEM4Z (Promega). ³²P-labeled tRNAs of high and low specific activity were prepared by run-off transcription as described (10).

Western and Northwestern Blots. Complexes resolved by non-denaturing electrophoresis were denatured *in situ* with 25 mM Tris, 190 mM glycine, and 0.05% SDS before semidry transfer to nitrocellulose (Hybond C, Amersham Pharmacia). 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 ³²P-labeled tRNA as described (29).

Photochemical Crosslinking. T7 RNA polymerase transcripts were doubly labeled with [α -³²P]UTP and 5-bromo-UTP and crosslinked to protein as described (12). RNA-protein binding reactions were performed in binding buffer (8) with probe RNA (1 nM) and concentrated dodecyl maltoside extract (from \approx 140 μ g of mitochondria) for 30 min on ice, then irradiated with 313-nm ultraviolet for 0.5–1 h.

Electrophoretic Mobility Shift Assay. Unless otherwise stated, ³²P-labeled tRNA (10 fmol) was incubated with refolded recombinant protein (50 fmol) for 10 min at 4°C in 5 μ l of binding buffer (8) and electrophoresed on native 4–15% gradient polyacrylamide gels (12) before autoradiography. K_d values of the RNA-protein complex were estimated by Scatchard analysis, as described (25).

Import Assays. Purified RIC (100 ng) was incorporated into phosphatidylcholine vesicles (50 μ g lipid), as described (12) and incubated with ³²P-labeled tRNA (100 fmol) and 4 mM ATP, and uptake was analyzed by RNase protection (12). Where indicated, low specific activity effector tRNAs were present at 1/10 the concentration of the high specific activity substrate tRNA.

Import Reconstitution. Before reconstitution, recombinant RIC1/F1 α (120 ng in 6 μ l), refolded as above, was diluted 5-fold into

BAM buffer, kept on ice for 2 h, further diluted to 200 μ l with buffer DB (0.2 M Tris-HCl, pH 7.5/5 mM MgAc₂/1 mM DTT/0.1 mM PMSF/10% glycerol), and concentrated down to 30 μ l by using a Microcon 10 ultrafilter (Amicon). Crude mitochondrial fractions prepared from 2×10^7 uninduced or RIC1-knockdown cells were extracted with BAM buffer, similarly diluted with buffer DB, and concentrated. Liposomes (50 μ g of lipid in 10 μ l of DB) were incubated with mitochondrial extract (8 μ l) with or without concentrated RIC1/F1 α (8 ng in 2 μ l) for 1 h on ice before import assay.

RNA End-Labeling. 5' Phosphate containing total mitochondrial tRNA was dephosphorylated with shrimp alkaline phosphatase, ethanol precipitated and 5'-labeled with [γ -³²P]ATP in presence of T4 polynucleotide kinase.

Mitochondrial Translation Assay. Washed cells (10^8) were suspended in 100 μ l of methionine-deficient RPMI medium 1640 (prepared by using the Invitrogen Select-Amine kit) containing cycloheximide (100 μ g/ml) and, where indicated, chloramphenicol (100 μ g/ml), incubated at 22°C for 15 min, then 40 μ Ci of [³⁵S]methionine (1,000 Ci/mmol; 1 Ci = 37 GBq) was added, and incubation continued for 4 h at 22°C with shaking. Mitochondrial fractions recovered from the labeled cells were treated with DNase and RNase, and mitochondrial proteins were solubilized in SDS/PAGE sample buffer for 1 h at 37°C before electrophoresis and fluorography.

Assays of Respiratory Function. Oxygen uptake and COX assays were performed as described in *Supporting Text*.

We thank the W. M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia, Charlottesville) for peptide analysis, George Cross (The Rockefeller University, New York) for the trypanosome targeting plasmids, the Coli Genetic Stock Center (Yale University, New Haven, CT) for the *atp A* strains, and Tapas Chowdhury for technical assistance. This work was supported by Department of Science and Technology Grant SR/SO/BB-28/2003, Council of Scientific and Industrial Research (CSIR) Project SMM 003, CSIR Fellowships (to S.G., G.D., B.M., S.M., and P.H.), and a University Grants Commission fellowship (to S.C.).

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Editorial Expression of Concern and Correction

BIOCHEMISTRY

PNAS is publishing an Editorial Expression of Concern regarding the following article: "A bifunctional tRNA import receptor from *Leishmania* mitochondria," by Srikanta Goswami, Gunjan Dhar, Saikat Mukherjee, Bidesh Mahata, Saibal Chatterjee, Pratik Home, and Samit Adhya, which appeared in issue 22, May 30, 2006, of *Proc Natl Acad Sci USA* (103:8354–8359; published ahead of print May 19, 2006, doi:10.1073/pnas.0510869103).

The editors wish to note that a reader has raised questions about the apparent duplication of certain figures in this article. We have reviewed the matter and are concerned about the validity of the results. The matter has been forwarded to the Indian Institute of Chemical Biology (IICB) to investigate further. We are awaiting the findings of IICB to determine the appropriate next steps.

Randy Schekman
Editor-in-Chief

www.pnas.org/cgi/doi/10.1073/pnas.1004225107

BIOPHYSICS AND COMPUTATIONAL BIOLOGY

Correction for "Evolution-guided discovery and recoding of allosteric pathway specificity determinants in psychoactive biogenic amine receptors," by Gustavo J. Rodriguez, Rong Yao, Olivier Lichtarge, and Theodore G. Wensel, which appeared in issue 17, April 27, 2010, of *Proc Natl Acad Sci USA* (107:7787–7792; first published April 12, 2010; 10.1073/pnas.0914877107).

The authors note that the e-mail address for Olivier Lichtarge should have appeared as lichtarge@bcm.edu.

www.pnas.org/cgi/doi/10.1073/pnas.1005260107