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A mosaic of RNA binding and protein interaction motifs in a bifunctional mitochondrial tRNA import factor from *Leishmania tropica*

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ABSTRACT

Proteins that participate in the import of cytosolic tRNAs into mitochondria have been identified in several eukaryotic species, but the details of their interactions with tRNA and other proteins are unknown. In the kinetoplastid protozoon *Leishmania tropica*, multiple proteins are organized into a functional import complex. RIC8A, a tRNA-binding subunit of this complex, has a C-terminal domain that functions as subunit 6b of ubiquinol cytochrome c reductase (complex III). We show that the N-terminal domain, unique to kinetoplastid protozoa, is structurally similar to the appended S15/NS1 RNA-binding domain of aminoacyl tRNA synthetases, with a helix–turn–helix motif. Structure-guided mutagenesis coupled with *in vitro* assays showed that helix α1 contacts tRNA whereas helix α2 targets the protein for assembly into the import complex. Inducible expression of a helix 1-deleted variant in *L. tropica* resulted in formation of an inactive import complex, while the helix 2-deleted variant was unable to assemble *in vivo*. Moreover, a protein-interaction assay showed that the C-terminal domain makes allosteric contacts with import receptor RIC1 complexed with tRNA. These results help explain the origin of the bifunctionality of RIC8A, and the allosteric changes accompanying docking and release of tRNA during import.

INTRODUCTION

In a large number of species, cytosolic tRNAs are imported into mitochondria, primarily to substitute for mutant or deleted organelle-encoded tRNAs (1,2). In the flagellated protozoa belonging to the order Kinetoplastidae, the total lack of mitochondrial tRNA genes necessitates large-scale tRNA import. Although the evolutionary significance of tRNA import is a matter of debate, recent studies identifying protein factors involved in this process have revealed that a significant number are documented or suspected bifunctional proteins with known additional roles in other metabolic pathways. The current list includes mitochondrial protein import factors in yeast and plants (3,4), enolase in yeast (5), the Voltage Dependent anion Channel (VDAC) in Arabidopsis (4), a translation factor in *Trypanosoma brucei* (6) and several components of oxidative phosphorylation (OX PHOS) complexes in the kinetoplastid protozoon *Leishmania* (7–10). Thus, the mechanism of bifunctionalization is of importance to the understanding of the evolution of mitochondrial tRNA import.

We have previously purified a multisubunit complex (RNA Import Complex or RIC) from inner mitochondrial membranes of *Leishmania tropica* that actively transports tRNA across natural and artificial membranes (11). Mitochondrial extracts from other kinetoplastid protozoa display complexes resolved by native electrophoresis in the size range of RIC that are apparently aggregates of complex V (12,13); such aggregates may mask the import complex, or the latter may be unstable to the isolation procedures, accounting for its apparent absence from these preparations, but this issue is yet to be resolved. Biochemical characterization, coupled with knockdown of individual subunits, showed that the *Leishmania* RIC is made up of 14–15 polypeptides encoded by 8 nucleus- and 2 mitochondrion-encoded genes (9). Five of the nucleus-encoded subunits are each specified by single or duplicated genes and shared with one other OX PHOS complex, indicating bifunctionality. Moreover, structural comparisons of the import factors with the corresponding conserved OX PHOS subunit in other species revealed the occurrence of extra sequences or domains at the N- or C-terminus of the protozoal protein (7–9). It is a reasonable hypothesis that these extra domains have import-related functions that do not interfere with the respiratory roles of the proteins, but formal proof of this is lacking, and the origins of the terminal extensions remain a mystery.

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RIC8A is a 21-kDa tRNA-binding component of the complex that is partly homologous to subunit UCR6b of ubiquinol-cytochrome c reductase (OX PHOS complex III) (8). The homology is restricted to the C-terminal region of RIC8A. The N-terminal extension of ~80 amino acid residues has autonomous tRNA-binding activity (8), and is absent from UCR6b of other species, but its structure is yet to be determined. RIC8A has binding specificity for the so-called type II tRNAs which, *in vitro*, require stimulation by a second group of tRNAs (type I) for import across the inner mitochondrial membrane; type I tRNAs, on the other hand, are imported efficiently by themselves, but this import is inhibited by type II tRNAs (11,14). These effects are due to reciprocal allosteric interactions between the type I and type II receptors (RIC1 and RIC8A, respectively), bound to their respective tRNA ligands. Knockdown of RIC8A leads to deficiency of mitochondrial type II and elevation of type I tRNAs *in vivo* (8). In the presence of ATP, both types of tRNA are transferred from their respective receptors to a third subunit, RIC9, and thence through the membrane (15). The phenomenon of allosteric regulation of tRNA import has not been studied in other species of kinetoplastid protozoa. However, all of the *L. tropica* RIC subunit genes are conserved in other kinetoplastid species, and the corresponding proteins are nearly or completely identical in sequence across species (9). Thus, there is no reason to exclude the possibility of occurrence of allosteric regulation in these species also.

Taken together, these results imply the occurrence of multiple stable and/or transient interactions of RIC8A with tRNA and with other subunits of the import complex that are coordinated to ensure that the protein is differentially assembled into the import complex and complex III; and secondly, that tRNAs are docked and released directionally during import, but the details of such interactions remain to be elucidated. Here we have examined through homology modeling the structural relationship, if any, between the N-terminal domain of RIC8A and other known tRNA binding domains. Structure-guided mutants of the domain were tested for effects on respiratory function or import, and for assembly and tRNA-binding properties.

MATERIALS AND METHODS

Homology modeling

Gene and protein sequences were retrieved from the *Leishmania major* genome sequence database (http://www.genedb.org/genedb/leish/index.jsp). The N-terminal extended region of RIC8A (residues 1–82) or LysRS was modeled on the solution nmr structure of the hamster EPRS second repeated element (ExPDB code 1d2dA) in the SwissModel server (www.expasy.ch/swissmod/SWISS-MODEL.html). Published sequences (listed in ref. 16) of the S15/NS1 RNA-binding domain (SCOP classification, Protein Data Bank) were aligned with the modeled RIC8A structure, using the First Approach Mode with User-defined Template. The program identifies the template on the basis of sequence identity with the query (minimum 25%), rejects models smaller than 20 residues and performs energy minimization. At 30–39% identity, the reliability of the model, i.e. accuracy to within 5 Å, is estimated to be 77% (cf. the Swiss Model server).

Deletion and point mutagenesis

The cloning of the RIC8A gene has been described (7). RIC8A fragments encompassing different structurally defined domains were PCR-amplified using appropriate sense and antisense primers (Table S1) from the parental clone of full-length gene, and inserted into the appropriate expression vector. The complete RIC8A gene includes a putative cleaved N-terminal mitochondrial targeting sequence [MTS; (8)]. Unidirectional deletions from the N terminus (mutants C, D1, D2 and D3; Figure 1A) lack the MTS; therefore, to express these proteins in *Leishmania* mitochondria, the PCR-amplified fragments were fused to the MTS (amino acid residues 1–30) of subunit RIC1 (7) in vector pUC(MTS-1) (Dhar,G. and Adhya,S. manuscript in preparation). The remaining clones (N, D4–D6, H1 and H2) contain the endogenous MTS of RIC8A. To construct internal deletions D4–D6, the corresponding amplified fragments were fused in-frame to the N-terminus of clone C (containing residues 82–202); this resulted in the creation of a 2-residue linker between the two fused domains. Internal deletions H1 and H2 were similarly constructed by fusing deletion fragments D6 and D5 with the N-terminus of fragments D2 and D3, respectively (Figure 1A). For bacterial expression, fragments were cloned downstream of the glutathione-*S* transferase (GST) gene of pGEX4T-1 (GE Life Sciences). For expression in *Leishmania*, the inserts were transferred in the sense orientation from pGEX4T-1 to expression vector pGET, between its HindIII and BamHI sites (7). Alanine-scanning point mutations P1–P7 were constructed using the wild-type RIC8A N-terminal domain (residues 1–82; derivative N in Figure 1A), cloned into the BamHI/EcoRI sites of pGEX4T-1, as template. Point mutagenesis was performed using PCR with mutagenic primers according to the method of Ito *et al.* (17). The mutant PCR-amplified fragment was cloned into pGEX4T-1. Details of mutagenesis protocols are provided in Supplementary Data. For expression in *Leishmania*, the relevant derivatives were excised from pGEX4T-1 or pUC19 backbone and inserted between the HindIII and BamHI sites of vector pGET, downstream of the inducible T7 RNA polymerase promoter.

Protein expression

Gene fragments were expressed as GST fusion proteins in *Escherichia coli* from the pGEX4T-1 clones described above. The fusion proteins were cleaved with thrombin and the expressed peptides were separated by gel electrophoresis, as detailed elsewhere (7).

RNA-binding assays

^{32}P-labeled tRNA transcripts were generated by T7 polymerase-mediated transcription of cloned *Leishmania* tRNA genes (7). Indicated amounts of recombinant protein and radiolabeled tRNA were incubated in

10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT and 0.1 M KCl) for 30 min on ice. Complexes were separated from unbound tRNA by 5–15% native PAGE (11) and detected by autoradiography. To determine dissociation constant (K_d), a constant amount of protein was titrated with radiolabeled RNA. Bands were quantified densitometrically and Scatchard plots of (bound)/(free) versus bound tRNA (8) were constructed.

Gene knockdown and overexpression

Plasmid pGET(RIC8A-AS) contains the complete coding sequence of *Leishmania* RIC8A cloned in antisense orientation downstream of a tetracycline-inducible promoter of targeting vector pGET carrying a BLE (bleomycin-resistance) marker gene (8). In overexpressing constructs, relevant gene fragments [containing the original MTS or fused with the MTS of RIC1 (see above)] were cloned in the sense orientation in pGET. The plasmids were transfected into the host strain *L. tropica* 13–90 expressing the tetracycline repressor and T7 RNA polymerase, and transformants selected by phleomycin resistance (7). Individual clones were grown and antisense RNA, or mRNA, induced with 1 μg/ml tetracycline. Analysis of mitochondrial complexes, respiration or tRNA was performed on cells at the earliest point of growth arrest (2–3 days of tetracycline treatment) or after 5 days (in the absence of a growth effect).

Complex analysis

Inner membrane macromolecular complexes in sodium dodecyl maltoside-solubilized mitochondrial extracts (~100 μg protein) were resolved by Blue Native (BN) electrophoresis, as described (7), and visualized by Coomassie blue staining, or probed with specific antibodies by western blotting. For second-dimension analysis, individual bands from the BN gel were excised, denatured *in situ*, and the subunits separated by Tricine SDS 16% PAGE (18); this system is particularly suitable for resolving low-molecular-weight proteins e.g. between the wild-type RIC8A (202 residues), H1 (195 residues) and H2 (185 residues) (Figure 5).

In vitro assembly

Mitochondria from 2×10^7 cells were extracted with 50 mM Bis Tris–HCl pH 7.0, 0.75 M aminocaproic acid, 2% dodecyl maltoside, diluted to 200 μl with 0.2 M Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 10% glycerol and concentrated by Microcon 10 ultrafiltration. Concentrated extract (8 μl = 2×10^6 cell equivalent) was combined with recombinant RIC8A or its derivative (8 ng/2 μl) and phosphatidyl choline vesicles (50 μg lipid in 10 μl) for 1 h at 4°C before analysis by BN PAGE or import assay (11).

Analysis of mitochondrial tRNAs

Northern blots of ~5×10^6 cell-equivalents of mitochondrial RNA were probed with ^{32}P-labeled oligonucleotides (Table S1) complementary to specific tRNAs.

In vitro import assay

Reactions (20 μl) contained 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM ATP, 5 nM ^{32}P-labeled tRNA substrate labeled to high specific activity, 0.5 nM low-specific-activity tRNA effector (where indicated), and reconstituted liposomes (50 μg lipid, 100 μg mitochondrial protein), for 15 min at 37°C, then treated with RNase, and the RNase-resistant RNA recovered and analyzed by urea–PAGE, as described (11).

Protein interaction assay

Binding reactions of RIC1 with RIC8A N-terminal and C-terminal domains in presence or absence of $tRNA^{Tyr}$ were performed as follows. ^{32}P-labeled $tRNA^{Tyr}$, purified recombinant N-terminal and RIC8A C-terminal proteins were incubated in equimolar ratios (1 pmol each) in 10 μl reactions containing 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 M KCl, for 45 min on ice and then electrophoresed on native 4–15% gradient polyacrylamide gels, as described (11). The gel was Coomassie-stained, western-blotted and probed with antibody, or autoradiographed.

Western blotting

Proteins resolved by gel electrophoresis were electroblotted to Hybond C membranes (GE Life Sciences) and the blots were probed with a polyclonal antiserum against purified, recombinant RIC8A, as described (8). This antiserum recognizes epitopes throughout the protein, enabling the detection of all isolated domains. Bands were visualized by the alkaline phosphatase (AP) color reaction catalyzed by AP-conjugated secondary antibody. This is a sensitive but nonquantitative method, i.e. the signals are not proportional to the amount of specific protein.

Immunoprecipitation

Leishmania tropica promastigotes were cultured overnight in methionine-deficient medium RPMI 1640 (Invitrogen) with ^{35}S-methionine 1000 Ci/mmol, 100 μCi/ml). Harvested, washed cells were extracted with phosphate-buffered saline (PBS) containing 0.2% SDS. The extract was diluted 20-fold into TETN buffer [250 mM Tris–HCl (pH 7.5), 5 mM EDTA, 250 mM NaCl, 1% Triton X-100, 2 mg of BSA/ml] containing nonimmune or anti-RIC8A antiserum (1:50). Antigen–antibody complexes were recovered with protein A-Sepharose beads (Sigma) and run on SDS–PAGE. The gel was subjected to fluorography to sensitively visualize the bands. Exposures times of the films were adjusted to be within the proportional range. Pixel densities on scanned, inverted images were determined for each band, adjusted for the neighboring background and used as a quantitative measure of band intensities.

RESULTS

Structural similarity between the N-terminal region of RIC8A and the S15/NS1 RNA-binding domain

Although the C-terminal 120-residue region of RIC8A structurally overlaps with UCR6b (8), the relationship

between the RNA-binding site in the N-terminal 80-or-so residues and known tRNA-binding domains is unknown. During an *in silico* search for known structures on which the N-terminal sequence could be threaded to form a homology model, we found significant overlap with the S15/NS1 domain (variously termed recurrent, appended or helical, domain) appended to several eukaryotic aminoacyl tRNA synthetases (aaRS) (Figure 1B). This domain (16) consists of two antiparallel α-helices, α1 and α2, connected by a linker. In RIC8A, the homologous regions map to residues 36–49 (α1) and 54–64 (α2). An overlapping structure was found in a single aaRS in *Leishmania*, LysRS (Figure 1), but there was considerable divergence at the level of primary sequence. The reliability of such models depends on the sequence similarity between the query and the template; in the case of RIC8A N-terminal sequence the sequence similarity with the hamster EPRS S15/NS1 domain is 39% (Figure 1C), corresponding to an estimated reliability (accuracy to within 5 Å) of nearly 80% (www.expasy.ch/swissmod/SWISS-MODEL.html). Importantly, the energy-minimized structure generated working hypotheses on the role of individual regions or residues, to be confirmed or denied by site-directed mutagenesis.

Comparison of the sequences showed divergence of the RIC8A S15/NS1-like domain from the consensus aaRS appended domain (Figure 1C). The α1 helix of RIC8A retains the two conserved basic residues of the aaRS domain, but a third such residue (Lys42) appears at a nonconserved position, generating a tandem array of 3 Lys/Arg spaced 3 residues apart, i.e. on the same side of the helix. Additionally, a conserved valine immediately upstream of the second Arg in this array has been replaced by Glu. In helix α2, two conserved hydrophobic residues (Ile and Val) have been replaced by Lys57 or Arg61, respectively; conversely, two conserved charged residues (Asp/Glu and Lys) have been substituted by hydrophobic residues. These positions provided targets for structure–function studies by site-directed mutagenesis (see below).

The α1 helix is critical for tRNA binding

RIC8A has intrinsic affinity for a subset of tRNAs—the type II tRNAs—that are imported into mitochondria (8). To localize the tRNA-binding site within the N-terminal domain, deletion and point mutations were generated (Figure 1A), the derivatives expressed in *E. coli*, and the recombinant proteins tested for binding to $tRNA^{Ile}$, a type II tRNA (14), by gel-shift assay. Mutant D1 in which the N-terminal 33 residues had been deleted was active for tRNA binding, whereas D2 and D3, with N-terminal deletions mapping to residues 46 and 66, respectively, were inactive (Figure 2). Mutants D4–D6 have internal deletions with the same right endpoint (residue 81) and different left endpoints (residues 71, 50 and 40,

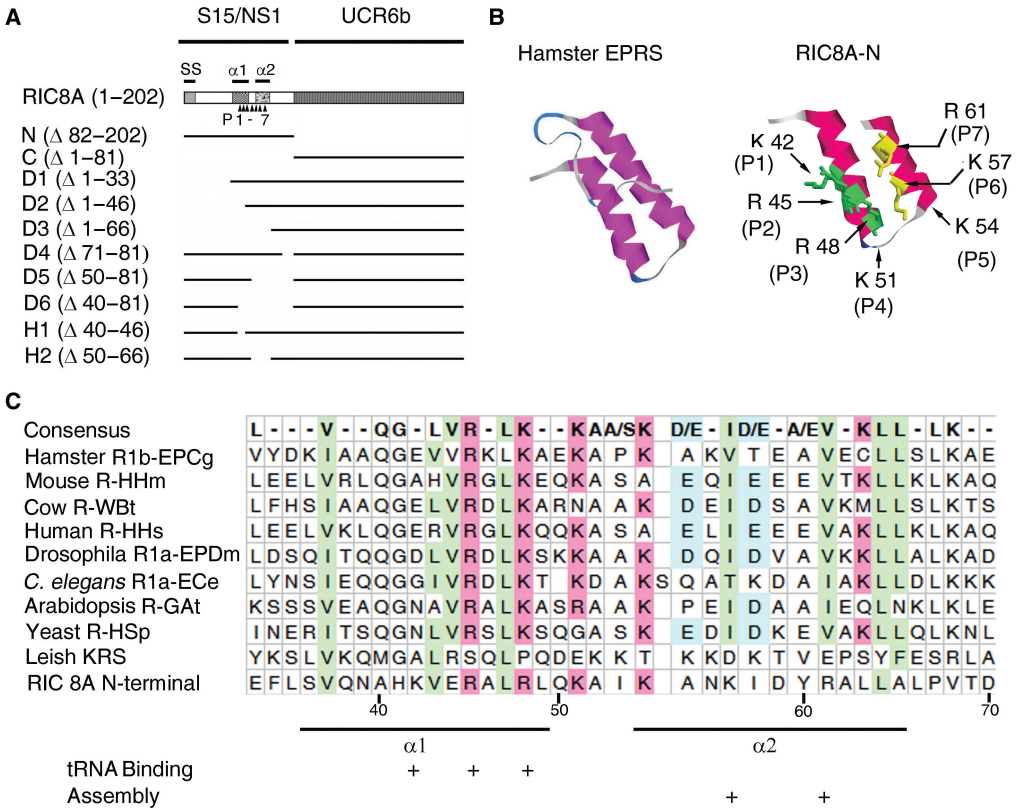

Figure 1. Domain structure and organization in RIC8A. (**A**) Domains and subdomains: ss, mitochondrial targeting sequence; α1, α2, the two helices of the S15/NS1-like domain. Positions of various deletions (D) and point mutations (P) are shown. (**B**) Homology model of RIC8A N-terminal domain (right) templated on the hamster GluProRS repeat R1. Side chains of residues altered to alanine are indicated (mutant numbers in parenthesis). (**C**) Sequence alignment of the α1–α2 region of RIC8A with the appended S15/NS1 domain of eukaryotic aaRS. Different residue types are shaded differently: pink, basic; blue, acidic; green, hydrophobic. Residues involved in tRNA binding, or assembly, are indicated by + signs.

respectively). D4 and D5 had the normal affinity for $tRNA^{Ile}$, whereas D6 was inactive (Figure 2B). Taken together, these results indicate that the region between residues 33 and 50 is critical for tRNA binding, which is almost coincident with the position of helix α1 (residues 36–49). This helix contains three basic amino acid residues spaced 3 residues apart, and therefore oriented on the same side of the helix (Figure 1). Point mutants K42A, R45A and R48A, and deletion mutant H1 lacking helix α1, all had reduced or abolished tRNA-binding activity (Figure 2B); quantitative analysis showed an ~4-fold increase in the apparent dissociation constant (K_d) of the complex in each case, compared to the wild-type protein (Figure 2C and D). In contrast, tRNA binding was not affected in the point mutant K51A, in the linker region, or in mutants K54A, K57A and R61A, or in deletion mutant H2 (Figure 2B and D), mapping to helix α2. These results confirm the role of the α1 helix in the interaction of RIC8A with tRNA. Moreover, each positive residue located on the hydrophilic surface contributes to the docking of the tRNA substrate. Thus, the divergence of the RIC8A domain to Lys42 has resulted in increase in the RNA-binding affinity (and perhaps specificity) of the protein.

The α2 helix is required for assembly of RIC8A into the import complex

The above mutants were also checked for their ability to be assembled into the import complex *in vitro*. The assay is based on the premise that subunits are assembled in an ordered sequence; thus, the deficiency of a particular subunit (generated by gene knockout or knockdown) will result in the accumulation of a partially assembled subcomplex in mitochondria. Addition of the purified subunit to the knockdown mitochondrial extract will then result in rebuilding of the complex with incorporation of that subunit as well as those downstream of it in the assembly pathway. This approach, combined with the use of subunit-specific antibodies, indicated that RIC8A directly contacts the 19-kDa subunit RIC9 during assembly (15).

In normal *L. tropica*, four mitochondrial complexes—RIC, and respiratory complexes III, IV and V can be resolved by BN electrophoresis [(7); Figure 3]. As described recently (15), complexes III and IV were absent in RIC8A-knockdown cells; complex V was truncated (species labeled V^{KD}; Figure 3A); and an assembly intermediate of RIC (R^{KD}), lacking various subunits in addition to RIC8A [(15); Figure 3D], was formed (Figure 3A). Addition of purified deletion fragments D1, D2 and D4 of RIC8A to the reconstitution reaction resulted in the specific retardation of the RIC subcomplex to form R^{RC} containing the added fragment (as shown by western blotting; Figure 3B), indicating this fragment to be competent for assembly. In contrast, deletions D3, D5 and D6 were not assembled. Considering the map positions of D1–D6 (Figure 1A), these results place the essential assembly motif between residues 46 and 71, roughly coinciding with the position of helix α2 (54–64). Moreover, mutant H1 (lacking residues 40–46) was assembly competent, and was detected in second-dimension gels of the reconstituted complex (Figure 3D), but H2 (lacking residues 50–66) or point mutants P6 (K57A) and P7 (R61A), both within helix α2, was not (Figure 3C).

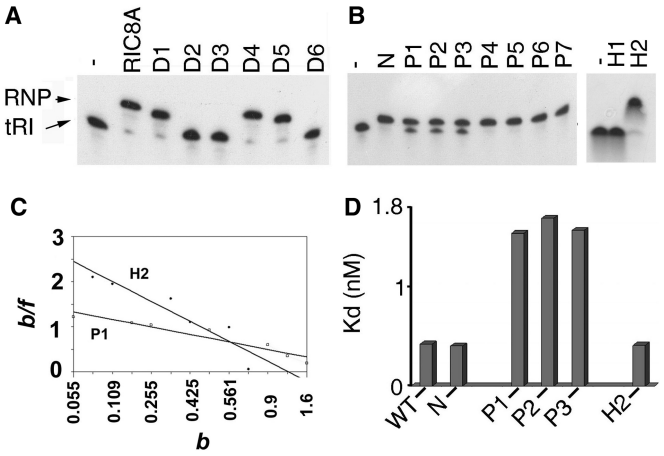

Figure 2. tRNA-binding activity of RIC8A mutants. (**A** and **B**) Gel-shift assays using ^{32}P-labeled $tRNA^{Ile}$ (tRI) (10 fmol) and wild-type (RIC8A) or indicated mutant recombinant proteins (100 fmol). RNP, ribonucleoprotein. (**C**) Examples of Scatchard plots for mutants H1 and P2. *b*, concentration of bound tRI; *f*, concentration of free RNA. (**D**) Dissociation constants (K_d, nM) of complexes of tRI with indicated RIC8A derivatives. All variants are recombinant proteins expressed in *E. coli*. P1–P7 are derived from clone N (Figure 1) spanning residues 1–82.

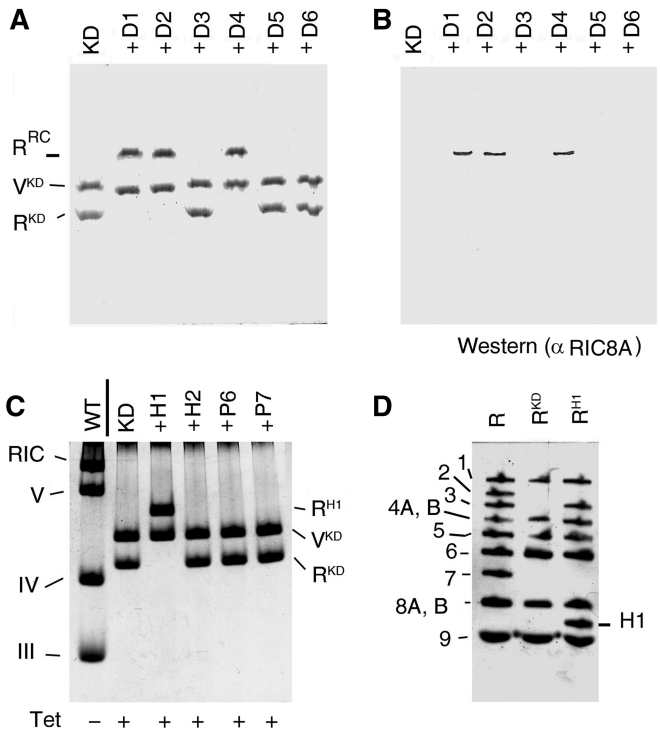

Figure 3. Assembly competence of RIC8A mutants *in vitro*. (**A** and **C**) BN PAGE of RIC8A-knockdown complex (KD) reconstituted with indicated mutant proteins. WT, complexes from uninduced cells, Coomassie stain. (**B**) Western blot of gel run in parallel to (A), probed with anti-RIC8A antiserum (1:50). The amount of protein loaded on this gel was about one-third that in (A). R^{KD}, knockdown RIC subcomplex; V^{KD}, complex V from KD cells; R^{RC}, reconstituted RIC. (**D**) SDS–PAGE profile of R^{KD} and $R^{RC\ (H1)}$ (RIC reconstituted with H1 protein), compared to wild-type RIC (R). RIC subunits are numbered as in ref. 9.

Thus, helix α2 appears to be critical for assembly of RIC8A into the import complex.

Distinct functions of the two α helices *in vivo*

To examine the *in vivo* role of helices 1 and 2 within the N-terminal domain, we constructed RIC8A derivatives H1 and H2 lacking these two helices (Figure 1), which were individually expressed in *L. tropica* from the expression vector pGET (7) under the control of a tetracycline-inducible T7 RNA polymerase promoter, and the inner membrane complexes were analyzed for the presence of the ectopically expressed protein; in parallel, the effects on import, respiration and the activities of respiratory complexes were determined. The rationale for this approach is that a derivative which is competent to assemble into RIC or complex III will compete with endogenous RIC8A for assembly into the corresponding complex; the downstream effects will then be a function of the activity of the ectopic derivative in the complex. On the other hand, a derivative that lacks a functional assembly motif will be unable to compete with the endogenous RIC8A, and therefore will be without effect on import or viability.

In cells overexpressing deleted RIC8A derivatives, an additional band representing the ectopic protein was detected by western blotting (Figure 5A–D, left-most panels), but this method, involving enzymatic color development, is nonquantitative. To estimate the extent of overexpression, quantitative immunoprecipitation of protein biosynthetically labeled with ^{35}S-methionine was performed (Figure 5A). This showed that the truncated protein was ~15-fold more abundant than the endogenous full-length RIC8A. This substantial extent of overexpression (10- to 20-fold for different proteins) accounts for the near-complete replacement of the wild-type protein by the ectopic variant in the appropriate mitochondrial complex (see below).

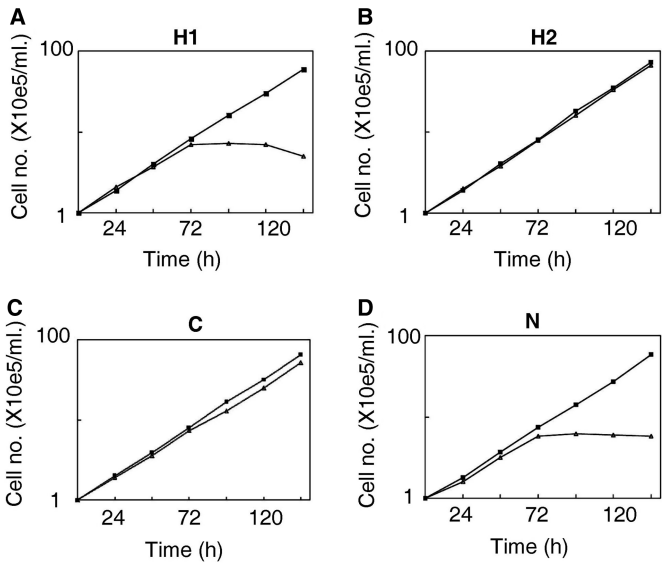

Figure 4. Growth curves of uninduced (squares) or tetracycline-induced (triangles) promastigotes transformed with RIC8A derivatives. (**A**–**D**) Deletion mutants H1, H2, C-terminal and N-terminal domain, respectively.

Expression of H1 in *L. tropica* resulted in growth arrest after ~72 h of tetracycline induction (Figure 4A) and reduction of O_2 uptake (Table S2). In mitochondria from cells harvested at the earliest point of growth arrest (72 h), the normal profile of 3 OX PHOS complexes and RIC was replaced by one resembling the knockdown profile, i.e. absence of complexes III and IV, and replacement of RIC and complex V by two subcomplexes R^{H1} and V^{H1}, respectively (Figure 5A). Under similar conditions of overexpression, the half-life of complex V is ~12 h

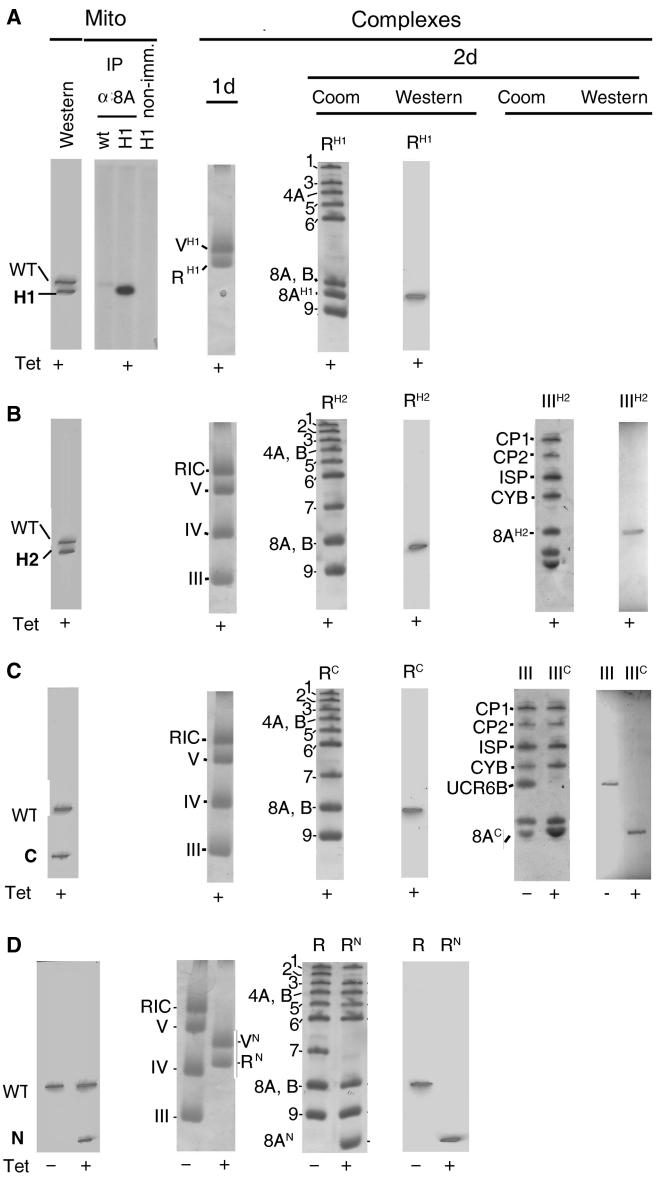

Figure 5. Effects of expressing RIC8A mutants on mitochondrial complexes of *L. tropica*. (**A**–**D**), Complexes in cells expressing H1, H2, C-terminal domain or N-terminal domain, respectively. Left to right: western blot of total mitochondrial protein resolved by Tricine–SDS–PAGE, and probed with polyclonal anti-RIC8A antibody; ^{35}S-methionine-labeled protein from wild-type or H1-expressing cells immunoprecipitated with nonimmune or anti-RIC8A serum; BN–PAGE profiles (Coomassie stain and western blot) of mitochondrial complexes; and subunit profiles (SDS–PAGE) of RIC and complex III. Subunit numbering as in ref. 9. Sizes of the endogenous (WT), H1 and H2 are 202, 195 and 185 amino acid residues, respectively.

(Dhar,G. and Adhya,S. unpublished data), accounting for the near-complete disappearance of this complex by the time of growth arrest. Second-dimension analysis showed that the ectopic H1 replaced endogenous RIC8A in the RIC subcomplex (Figure 5A), and is thus competent for assembly.

Northern blot analysis (Figure 6A) of nucleus-encoded mitochondrial tRNAs from normal or uninduced cells (WT) revealed differences in the steady-state levels of individual tRNAs (e.g. $tRNA^{Ile}$ was significantly more abundant than $tRNA^{Tyr}$); similar variations have also been observed in *T. brucei* mitochondria, and may reflect different import efficiencies (19). In H1-expressing cells the levels of some tRNAs, such as $tRNA^{Ile}$ and $tRNA^{Val}$, were significantly reduced or became undetectable, while those of other tRNAs, such as $tRNA^{Tyr}$ and $tRNA^{Arg}$, were elevated 2- to 2.5-fold (Figure 6A). These two groups of tRNAs were classified as type II and type I, respectively (12); thus, the import defect is typical of a nonfunctional type II receptor. This was verified by reconstituting liposomes with mitochondrial extracts from the overexpressing cells and assaying for *in vitro* import. Wild-type extracts induced import of the type I $tRNA^{Tyr}$ alone, but this import was inhibited by low concentrations of the type II effector $tRNA^{Ile}$; conversely, import of $tRNA^{Ile}$ required the presence of type I effector (Figure 6B).

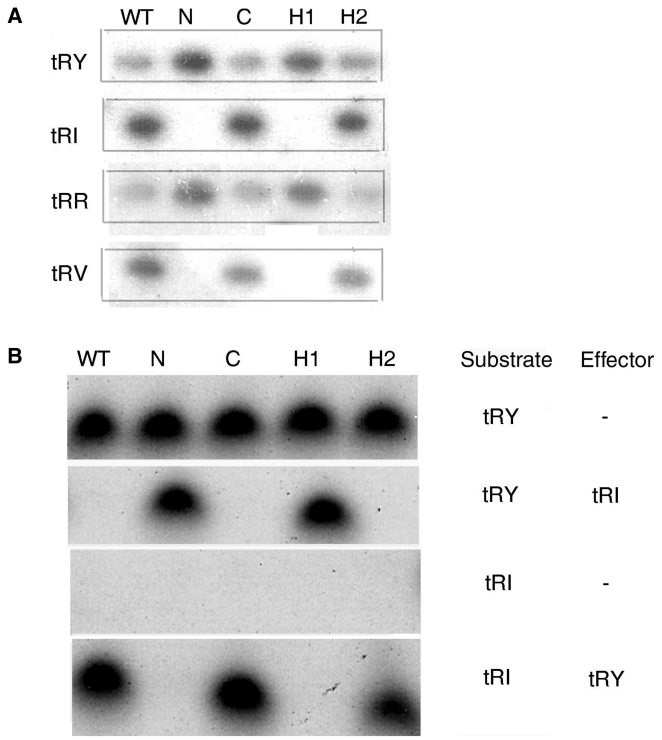

Figure 6. Effect of expressing RIC8A derivatives on tRNA import. (**A**) Nucleus-encoded mitochondrial tRNAs in normal (WT) or mutant RIC8A-expressing cells. Northern blot of mitochondrial RNA (5×10^6 cell-equivalents) probed with antisense oligonucleotides complementary to the indicated tRNAs: tR(X), tRNA specific for amino acid X (single-letter code). (**B**) Import of indicated ^{32}P-labeled substrates, in the presence or absence of low-specific-activity effector, into liposomes reconstituted with extracts from RIC8A derivative-expressing cells (RNase protection assay).

In contrast, extracts from H1-expressing cells were incompetent in type II import even in the presence of type I tRNA, while there was no inhibition of type I tRNA import by type II effector (Figure 6B).

Expression of H2 had no effect on cell growth (Figure 4B), respiration (Table S2), complex III activity (Table S3) or import (Figure 6); the ectopic protein was incorporated into complex III but not into RIC (Figure 5B). Thus, deletion of helix 2 resulted in an assembly defect for RIC but not for complex III.

An import-related activity of the C-terminal domain

Expression of the C-terminal domain of RIC8A had no significant effect on growth (Figure 4C), and the OX PHOS complexes looked normal, except for the replacement of the endogenous RIC8A/UCR6b by the ectopic domain (the UCR6b-homologous region of RIC8A) (Figure 5C). RIC in these cells had the normal subunit profile, containing the endogenous full-length RIC8A, indicating the inability of the ectopic C-terminal domain to compete for assembly (Figure 5C). Mitochondrial tRNAs were at normal levels in these cells (Figure 6A), and the extracts had normal type II tRNA import activity on liposomes (Figure 6B). Both respiration and complex III activity were normal, (Tables S2 and S3), reflecting the functionality of the C-terminal domain in complex III, and the lack of any effect of its expression on tRNA import.

In contrast, induction of expression of the N-terminal domain of RIC8A resulted in growth arrest after ~72 h (Figure 4D). In the growth-arrested cells, the profile of inner membrane complexes was altered: complexes III and IV disappeared, while complex V and RIC were replaced by two subcomplexes V^N and R^N, respectively (Figure 5D). Second-dimensional analysis of R^N, followed by western blotting, revealed that the endogenous RIC8A had been replaced by the ectopic N-terminal domain; additionally, the mitochondrion-encoded subunits 2 and 7 were absent (Figure 5D). V^N lacked the mitochondrion-encoded subunit A6 (data not shown). In these mitochondria, type II tRNAs were selectively depleted, with elevation of type I tRNA levels (Figure 6A). Moreover, the corresponding mitochondrial extract was inactive for type II tRNA import even in the presence of type I tRNA (Figure 6B). Thus, although the isolated N-terminal domain binds tRNA, and is assembled into the import complex, it is import-defective, implying a role for the missing C-terminal domain, either to stabilize the structure of the N-terminal domain, or for interactions with other subunits of the complex.

Role of the C-terminal domain in inter-receptor allosteric interactions

Within the import complex, tRNA binding by RIC8A is activated by type I tRNA (7). Type I tRNAs bind to receptor RIC1, and anti-RIC1 antibodies inhibit this binding (11). This suggests that RIC1, bound to type I tRNA, contacts RIC8A to activate the type II tRNA-binding site of the latter.

To directly observe the interaction between these two receptors, recombinant RIC1 was incubated with either the expressed N- or C-terminal domain of RIC8A in the absence or presence of the ^{32}P-labeled type I tRNA^{Tyr}. The products were resolved by native gel electrophoresis, and the components of each complex were identified by western blotting and autoradiography. In presence of tRNA^{Tyr}, RIC1 formed a binary complex tRY-1 (Figure 7, lane 1). Neither the N- nor the C-terminal domain reacted with the type I tRNA, as expected, since the former domain has binding specificity for type II tRNAs, while the latter does not have an RNA-binding site (Figure 7, lanes 2 and 3). Addition of the N-terminal domain to the binary complex did not affect the latter (Figure 7, lane 4), and this domain did not interact with RIC1 in the absence of tRNA^{Tyr} (Figure 7, lane 6). In the absence of tRNA^{Tyr}, the C-terminal domain failed to bind RIC1 (Figure 4, lane 7). However, in presence of the tRNA, a ternary complex containing it, as well as RIC1 and the C-terminal domain, was formed (Figure 7, lane 5). Thus, RIC8A is allosterically activated by interaction of its C-terminal domain with the tRNA–RIC1 complex.

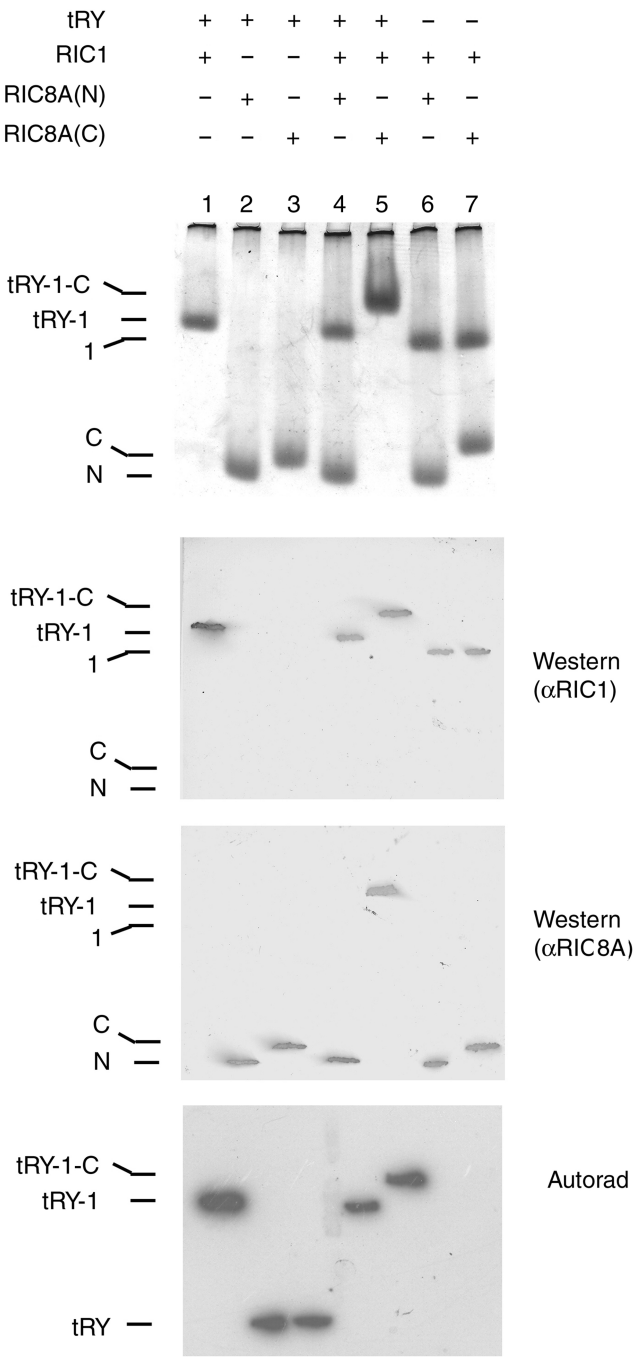

Figure 7. Interaction of the RIC8A C-terminal domain with RIC1. Recombinant proteins and ^{32}P-labeled tRNA^{Tyr} were incubated in the indicated combinations and the products separated by native gel electrophoresis. (**A**) Coomassie stain. (**B** and **C**) parallel western blots probed with anti-RIC1 or anti-RIC8A antibody, respectively. (**D**) Autoradiogram.

DISCUSSION

In this study, we have used a combination of *in vitro* and *in vivo* approaches to probe the various biological functions of RIC8A. We show how the native RIC can be remodeled *in vitro* as well as *in vivo*. One method involves efficient antisense-mediated knockdown of the native complex and its reconstitution *in vitro* with recombinant derivatives. Alternatively, the altered protein with an intact assembly motif can be overexpressed *in vivo*, to replace the wild-type protein by competition. Both approaches were facilitated by the development of an efficient tetracycline inducible expression system in *Leishmania* (7), allowing us to bypass the tedium of targeted gene replacement.

Our results point to critical roles of the extended N-terminal domain of the bifunctional UCR6b subunit of *Leishmania* OX PHOS complex III in mitochondrial tRNA import. Deletion of helix α1, or point mutations within this region, resulted in growth and tRNA import defects (Figures 4 and 6) attributable to a loss or reduction of tRNA-binding activity (Figure 2). Mutations or deletions in helix α2, on the other hand, did not affect tRNA binding but reduced or eliminated assembly of the protein into the import complex (Figure 3). Deletion of either helix will have obvious effects on the higher ordered structures of the N-terminal extension of RIC8A; thus the contribution of secondary as opposed to primary structure on tRNA binding or assembly activities cannot be evaluated on the basis of deletion analysis alone. However, the similar effects of point mutations (K/R to A) within the same region, expected to alter the secondary structure only minimally, emphasize the importance of side chain interactions.

The N-terminal domain of RIC8A is related to one of two ubiquitous and structurally distinct tRNA-binding domains that are appended to several aaRS (20). However, the S15/NS1 domain of GluProRS contains three such tandemly repeated domains and binds RNA nonspecifically (16). Although the appended domains may have originated as RNA chaperones, they may have undergone further structural and functional diversification during evolution. Thus, creation of the tandem repeat of positive charges on one face of helix α1 has contributed to

increases in specificity and binding affinity for tRNAs, while helix α2 has been modified for assembly into the import complex. We have shown elsewhere that RIC8A directly interacts with the 19-kDa subunit RIC9 during assembly of the complex, and that type II tRNA is transferred from the former to the latter during translocation in presence of ATP (15). A reasonable hypothesis is that helix α2 is involved in this interaction.

Although evolutionary changes in the ancestral helices 1 and 2 of the N-terminal domain may have led to increase in tRNA- binding affinity and appearance of a new protein-interaction motif, they are not sufficient to account for tRNA import function, since replacement of the full-length protein by the N-terminal domain within the complex resulted in inactivation of type II import (Figure 6). Therefore, for full functionalization, changes must have also occurred in the conserved C-terminal domain. This latter domain retains its respiratory function as a subunit of complex III, since replacement of the endogenous UCR6b by the ectopic C-terminal domain did not affect complex III assembly or function (Figure 6). However, it is also necessary for allosteric activation of type II tRNA import, probably mediated by its interaction with tRNA-bound RIC1 (Figure 7). Close inspection of the homology model of the RIC8A C-terminal domain revealed structural differences from the canonical UCR6b structure, e.g. the presence of an extra loop and a break in one of the α helices (data not shown), but whether these are functionally important remains to be determined.

Thus, RIC8A/UCR6b has at least four functional motifs within the overall two-domain structure: one each for type II tRNA binding (helix α1 of the N-terminal domain), for stable interaction with RIC9 (possibly helix α2), for allosteric interaction with RIC1 (in the C-terminal domain), and for interaction with complex III subunits (designated as motifs 1–4, respectively, Figure 8). The assembly and function of RIC8A can then be visualized as a series of conformational changes resulting in stable or transient protein–protein or protein–RNA interactions. Free RIC8A is presumed to be an equilibrium mixture of two mutually exclusive conformations with active motif 2 or 4; thus it can assemble in either the import complex or complex III, respectively. In the RIC-assembled protein, the tRNA-binding site (motif 1) remains closed. Activation of this site is contingent upon the binding of a type I tRNA to receptor RIC1, with conformational changes in the latter, leading to its interaction with motif 3 of RIC8A, and transmission of the structural perturbation to, and activation of, motif 1, and binding of type II tRNA. Subsequent to this, as detailed in our recent study, the tRNA is transferred to RIC9 and thence through the membrane, in well-defined steps driven by an ATP-generated proton-motive force (15).

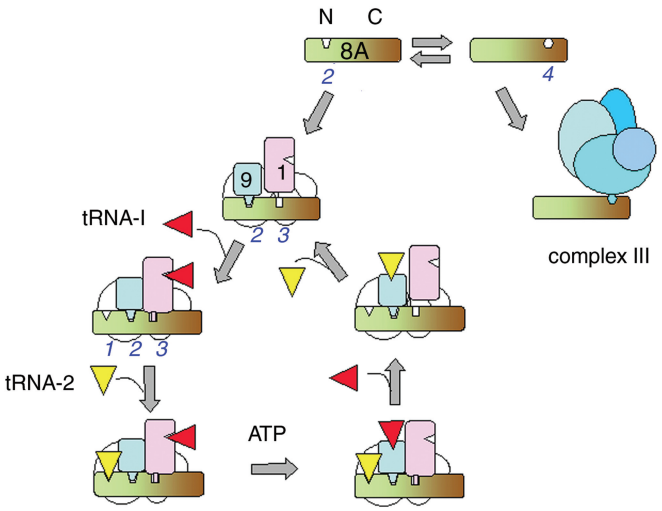

Figure 8. Schematic representation of the proposed role of the interaction motifs of RIC8A in assembly and import. Motifs 1–4 in RIC8A are lettered in blue. tRNA-I and tRNA-II are types I and II tRNAs, respectively. For details, see text.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Bhattacharyya,S.N. and Adhya,S. (2004) The complexity of mitochondrial tRNA import. *RNA Biol.*, **1**, 84–88.
2. Mirande,M. (2007) The ins and outs of tRNA transport. *EMBO Rep.*, **8**, 547–549.
3. Tarassov,I., Entelis,N. and Martin,R.P. (1995) An intact protein translocating machinery is required for mitochondrial import of a yeast cytoplasmic tRNA. *J. Mol. Biol.*, **245**, 315–323.
4. Salinas,T., Duchene,A.-M., Delage,L., Nilsson,S., Glaser,E., Zaepfel,M. and Marechal-Drouard,L. (2006) The voltage-dependent anion channel, a major component of the tRNA import machinery in plant mitochondria. *Proc. Natl Acad. Sci. USA*, **103**, 18362–18367.
5. Entelis,N., Brandina,I., Kamenski,P., Krasheninnikov,I.A., Martin,R.P. and Tarassov,I. (2006) A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in Saccharomyces cerevisiae. *Genes Dev.*, **20**, 1609–1620.
6. Bouzaidi-Tiali,N., Aeby,E., Charriere,F., Pusnik,M. and Schneider,A. (2007) Elongation factor 1a mediates the specificity of mitochondrial tRNA import in *T. brucei*. *EMBO J.*, **26**, 4302–4312.
7. Goswami,S., Dhar,G., Mukherjee,S., Mahata,B., Chatterjee,S., Home,P. and Adhya,S. (2006) A bi-functional tRNA import receptor from *Leishmania* mitochondria. *Proc. Natl Acad. Sci. USA*, **103**, 8354–8359.
8. Chatterjee,S., Home,P., Mukherjee,S., Mahata,B., Goswami,S., Dhar,G. and Adhya,S. (2006) An RNA-binding respiratory component mediates import of type II tRNAs into Leishmania mitochondria. *J. Biol. Chem.*, **281**, 25270–25277.
9. Mukherjee,S., Basu,S., Home,P., Dhar,G. and Adhya,S. (2007) Necessary and sufficient factors for import of tRNA into the kinetoplast-mitochondrion. *EMBO Rep.*, **8**, 589–595.
10. Adhya,S. (2007) *Leishmania* mitochondrial tRNA importers. *Int. J. Biochem. Cell Biol.* [Epub ahead of print; doi:10.1016/j.biocel.2007.10.025; October 25, 2007].

11. Bhattacharya,S.N., Chatterjee,S., Goswami,S., Tripathi,G., Dey,S.N. and Adhya,S. (2003) ''Ping-pong'' interactions between mitochondrial tRNA import receptors within a multiprotein complex. *Mol. Cell. Biol.*, **23**, 5217–5224.
12. 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) Characterization of the respiratory chain from cultured *Crithidia fasciculata*. *Mol. Biochem. Parasitol.*, **85**, 171–186.
13. Horvath,A., Kingan,T.G. and Maslov,D.A. (2000) Detection of the mitochondrially encoded cytochrome c oxidase subunit I in the trypanosomatid protozoan. *Leishmania tarentolae*. *J. Biol. Chem.*, **275**, 17160–17165.
14. Goswami,S., Chatterjee,S., Bhattacharyya,S.N., Basu,S. and Adhya,S. (2003) Allosteric regulation of tRNA import: interactions between tRNA domains at the inner membrane of *Leishmania* mitochondria. *Nucleic Acids Res.*, **31**, 5552–5559.
15. Basu,S., Mukherjee,S. and Adhya,S. (2008) Proton-guided movements of tRNA within the *Leishmania* mitochondrial RNA import complex. *Nucleic Acids Res.*, **36**, 1599–1609.
16. Cahuzac,B., Berthonneau,E., Birlirakis,N., Guittet,E. and Mirande,M. (2000) A recurrent RNA-binding domain is appended to eukaryotic aminoacyl-tRNA synthetases. *EMBO J.*, **19**, 445–452.
17. Ito,W., Ishiguro,H. and Kurusawa,Y. (1991) A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. *Gene*, **102**, 67–70.
18. Schagger,H. (2006) Tricine-SDS-PAGE. *Nat. Protocols*, **1**, 16–21.
19. Tan,T.H., Pach,R., Crausaz,A., Ivens,A. and Schneider,A. (2002) tRNAs in *Trypanosome brucei*: genomic organization, expression, and mitochondrial import. *Mol. Cell. Biol.*, **22**, 3707–3717.
20. Lee,S.W., Cho,B.H., Park,S.G. and Kim,S. (2004) Aminoacyl-tRNA synthetase complexes: beyond translation. *J. Cell Sci.*, **117**, 3725–3734.