

“Ping-Pong” Interactions between Mitochondrial tRNA Import Receptors within a Multiprotein Complex

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The mitochondrial genomes of a wide variety of species contain an insufficient number of functional tRNA genes, and translation of mitochondrial mRNAs is sustained by import of nucleus-encoded tRNAs. In *Leishmania*, transfer of tRNAs across the inner membrane can be regulated by positive and negative interactions between them. To define the factors involved in such interactions, a large multisubunit complex (molecular mass, ~640 kDa) from the inner mitochondrial membrane of the kinetoplastid protozoon *Leishmania*, consisting of ~130-Å particles, was isolated. The complex, when incorporated into phospholipid vesicles, induced specific, ATP- and proton motive force-dependent transfer of *Leishmania* tRNA^{Tyr} as well as of oligoribonucleotides containing the import signal YGGYAGAGC. Moreover, allosteric interactions between tRNA^{Tyr} and tRNA^{Ile} were observed in the RNA import complex-reconstituted system, indicating the presence of primary and secondary tRNA binding sites within the complex. By a combination of antibody inhibition, photochemical cross-linking, and immunoprecipitation, it was shown that binding of tRNA^{Ile} to a 21-kDa component of the complex is dependent upon tRNA^{Tyr}, while binding of tRNA^{Tyr} to a 45-kDa component is inhibited by tRNA^{Ile}. This “ping-pong” mechanism may be an effective means to maintain a balanced tRNA pool for mitochondrial translation.

There is remarkable diversity in the scope and mechanism of mitochondrial tRNA import (reviewed in reference 18). Human mitochondria do not import tRNA, but a number of neuromuscular degenerative and metabolic diseases are caused by mutations in mitochondrial tRNA genes (21). In yeast, a single tRNA is imported, apparently through protein import channels and requiring at least two soluble factors, including the mitochondrial form of the cognate aminoacyl-tRNA synthetase (8). By contrast, in kinetoplastid protozoa (*Leishmania* and trypanosomes), import of a whole spectrum of tRNAs is necessitated by the complete lack of mitochondrial tRNA genes (5, 19). In this system, membrane-bound tRNA binding proteins recognize specific structural motifs (import signals) on tRNA, soluble factors are not required, and the translocation pathway appears to be distinct from that for protein import (11, 14, 17). Moreover, the sequence and bioenergetic requirements for outer and inner membrane transfer are nonidentical (2), indicating the presence of a distinct transport machinery (the RNA import complex [RIC]) at the inner membrane, a situation similar to the TOM and TIM complexes for protein import (15). A 15-kDa polypeptide has been shown to be required for import into *Leishmania* mitochondria (1); otherwise, the import machinery remains undefined.

Using an in vitro evolution protocol, it was recently shown that *Leishmania* mitochondria recognize a number of short sequence motifs homologous to multiple domains in tRNAs, suggesting the presence of several import signals (3). Moreover, novel positive and negative allosteric interactions be-

tween these aptamers, as well as between intact tRNAs, at the inner membrane were described (3). The RNAs could be classified into two types: type I RNAs are efficiently transferred through the inner membrane but are inhibited by type II. In contrast, type II RNAs have poor inner membrane transfer efficiencies and are stimulated by type I. For example, tRNA^{Tyr} (GUA) is a type I RNA containing the conserved motif UA GAGC in the D domain, while tRNA^{Ile} (UAU) is type II with the sequence UCGCGGGUU in the variable loop-T domain (V-T) region (3). The mechanism of these allosteric interactions is unknown, but there are several possibilities. A single conformationally flexible dimeric or multimeric receptor could bind to either a type I or a type II motif. Alternatively, distinct type I and type II receptors may interact directly or indirectly through a third subunit. A related issue is whether the effector and substrate binding subunits for either RNA are identical or different.

To begin to define the molecular components of the inner membrane import apparatus involved in such allosteric interactions, we have isolated a protein complex from mitochondrial inner membrane that is functional for import and probed the role of individual subunits of the complex using a combination of chemical cross-linking and immunochemical approaches. The results reveal specific interactions between distinct type I and type II receptors at the inner membrane.

MATERIALS AND METHODS

Isolation of mitochondria. Mitochondria were prepared from *Leishmania tropica* strain UR6 and purified by Percoll gradient centrifugation (11). The outer membrane was solubilized by digitonin treatment, and the resultant mitoplasts were subjected to freeze-thaw cycles to separate particulate (inner membrane) and soluble (matrix) fractions (13).

Purification of RIC. Purified *Leishmania* mitochondria (20 mg of protein) were extracted with 320 μM digitonin as above, and mitoplasts were separated by

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centrifugation at $3,450 \times g$. Inner membranes were then prepared by three freeze-thaw cycles, suspended at 20 mg of protein/ml in sucrose-Tris-EDTA (STE) containing 0.5% sodium deoxycholate (DOC), 0.4 M KCl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM EGTA, and incubated on ice for 30 min with gentle agitation. The DOC extract was clarified by centrifugation at $16,000 \times g$ for 10 min. An RNA affinity column was prepared by immobilizing 500 pmol of RNA containing the D arm of tRNA^{Tyr} (see Fig. 3) and a 3'-terminal poly(A) tail on 0.1 ml of oligo(dT) cellulose and equilibrated with buffer DB (1) containing 0.1 M KCl. The DOC extract was diluted to 0.1 M in KCl with DB and loaded on the column, which was then washed sequentially with DB containing 0.125% DOC and 0.1, 0.25, 0.5, and 1.0 M KCl. The 0.5 and 1.0 M KCl fractions were concentrated by dialysis against dry Sephadex (1) or by centrifugal ultrafiltration. RNA binding activity was assayed by incubating the fractions with ³²P-labeled tRNA^{Tyr} in binding buffer (1) for 30 min on ice and observing the electrophoretic mobility shift of the RNA-protein complex in 6% polyacrylamide gel (60:1) in the presence of 5 mM MgAc₂. Polyclonal antibodies against individual bands resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were raised in BALB/c mice. The RIC21p gel band was trypsinized, and the digest was analyzed by liquid chromatography/mass spectrometry (MS) and MS/MS in a Finnigan LCQ ion trap mass spectrometer system. BLAST analysis was performed against the *L. major* sequence database (http://www.sanger.ac.uk/Projects/L_major/).

Reconstitution in phospholipid vesicles. Phosphatidylcholine (0.5 mg), dispersed in 150 μ l of liposome suspension buffer (50 mM HEPES-KOH [pH 7.5], 10 mM MgAc₂, 2 mM dithiothreitol [DTT], 10% glycerol, 0.25% DOC), was mixed with purified RIC or bovine serum albumin (BSA) (5 μ g in 50 μ l of DB-DOC-1 M KCl) and dialyzed against a mixture of 5 mM HEPES-KOH [pH 7.5], 5 mM MgAc₂, and 20 mM KCl for 18 h at 4°C (4). Aggregated material was removed by centrifugation at $2,300 \times g$, and the supernatant containing mainly unilamellar vesicles was used. Intactness of the vesicles was checked by observing the release of entrapped carboxyfluorescein in the presence of 1% Triton X-100. Washed proteoliposomes were analyzed by SDS-PAGE to reveal incorporation of RIC subunits (~36 μ g of protein/ml).

Import substrates. ³²P-labeled *Leishmania* tRNA^{Tyr}(GUA), tRNA^{Ile}(UAU), tRNA^{Gln}(CUG) and D arm oligoribonucleotides were prepared by T7 RNA polymerase transcription (1–3, 12). tRNA^{Ile} [42–66], covering positions 42 to 66 of the V-T region, was transcribed from a clone containing the internal *Hpa*II fragment of the tRNA^{Ile}(UAU) gene in plasmid pGCN8 (3). High- and low-specific-activity substrates and effectors for allosteric interaction assays were prepared as described previously (3). 5-Bromouridine (5-BrU)-labeled tRNA was synthesized in transcription reactions containing 10 μ M [α -³²P]UTP and 20 μ M 5-BrU triphosphate.

Import assays. Import into intact mitochondria or mitoplasts was assayed by incubating the vesicles (100 μ g of protein) with ³²P-labeled RNA (100 fmol) in 20 μ l of import buffer (10 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM DTT, and 4 mM ATP) at 37°C for 15 min; then, RNases A and T1 were added to final concentrations of 2.5 μ g/ml and 50 units/ml, respectively, and RNase digestion continued at 37°C for 15 min. The mitochondria were washed with STE by centrifugation, and imported RNA was isolated by guanidinium isothiocyanate extraction and ethanol precipitation (12). Proteoliposomes (~0.7 μ g of RIC) were incubated with 100 fmol of substrate RNA as above and treated with RNase; then, 3 ml of liposome washing buffer (5 mM HEPES-KOH [pH 7.5], 2 mM DTT, 5 mM EDTA) was added, and the vesicles were recovered by centrifugation at $120,000 \times g$ for 30 min at 4°C for RNA analysis. For allosteric interaction assays, proteoliposomes were incubated with a mixture of high-specific-activity substrate (50 fmol) and low-specific-activity effector (5 fmol) as indicated.

Electrophoretic and sedimentation analyses. Native RIC was electrophoresed on 4 to 15% gradient polyacrylamide (40:1) containing 50 mM Tris-HCl [pH 8.9], with 25 mM Tris-glycine as running buffer, at 8 V/cm. For Western blots, the gel was equilibrated with Tris-glycine-SDS buffer before transfer to a polyvinylpyrrolidone membrane. The blots were probed with mouse polyclonal anti-subunit serum at 1:100 dilution and developed with horseradish peroxidase-conjugated secondary immunoglobulin G. Sedimentation analysis was performed in 15 to 30% glycerol gradients in buffer DB at $150,000 \times g$ for 4.5 h at 4°C.

Electron microscopy. Freshly purified RIC (nondialyzed 1 M KCl fraction) was diluted to ~3 μ g/ml with buffer DB, and 10 μ l was applied on carbon-coated grids for 2 min; the grids were washed twice with deionized water and stained with 1% uranyl acetate for 2 min before observation at $30,000\times$. Photomicrographs were scanned on a Bio-Rad G710 densitometer. Particle size measurements were performed using Quantity One software.

Photochemical cross-linking and immunoprecipitation. Mitoplasts were incubated with 5-BrU-labeled tRNA (1 nM) for 30 min on ice, washed, and irradiated

with 313-nm UV on a transilluminator for 30 min with cooling to form RNA-protein cross-links (20). SDS was then added to 0.2%, and the proteins were diluted 20-fold into $1\times$ 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 a 1:50 dilution of polyclonal mouse antiserum. Immunoprecipitation was performed with protein A-Sepharose. Immune complexes were dissociated in SDS-PAGE sample buffer and resolved by 8 M urea-6% PAGE in the presence of 0.05% SDS. Antibody-inhibition experiments were performed on mitoplasts as described previously (1).

RESULTS

A large complex from the inner membrane. To purify the RIC, we exploited the observation that the D domain of *Leishmania* tRNA^{Tyr}(GUA) contains a necessary and sufficient import signal (12). A DOC extract of inner membrane preparations was chromatographed on an affinity column containing the D arm oligoribonucleotide (see below) as an affinity ligand. By gel-shift assay using the crude extract, a specific ribonucleoprotein complex containing tRNA^{Tyr} could be discerned (Fig. 1). The activity was retained by the affinity column and was specifically eluted with 1 M K⁺. This step resulted in a 40-fold enrichment of the protein in greater-than-80% yield (Table 1).

SDS-PAGE analysis of the 1 M K⁺ fraction showed the presence of 10 major bands ranging from 19 to 62 kDa, present in the apparent stoichiometry of 2:3:1:3:1:1:1:1:2:1, respectively, with an aggregate molecular mass of ~620 kDa (Fig. 1). The profile, though complex, is reproducible, with correspondence between the Coomassie- and silver-stained bands (Fig. 1). A number of substoichiometric bands were also present; these could be contaminants or proteolytic degradation products. Native gel electrophoresis of the affinity-purified fraction revealed the presence of a single protein species migrating with an apparent molecular mass of 640 kDa (Fig. 2). Sedimentation analysis yielded a value of ~620 kDa (Fig. 2). Glycerol gradient centrifugation resulted in a 50% increase of specific activity over the affinity fraction but a relatively low yield (Table 1), probably due to inactivation during sedimentation. The peak protein fraction in the glycerol gradient was sufficient to induce import, and no other fraction contained activity (data not shown), indicating the absence of smaller essential components; however, the possibility of multiple large complexes of identical size cannot be presently excluded. Western blot analyses demonstrated the presence of several of the subunits, including the 52- and the 21-kDa species, as well as one or more proteins binding specifically to tRNA^{Tyr}, but not to tRNA^{Gln}(CUG), which is not imported, in the native 640-kDa complex (Fig. 2).

Electron microscopy of negatively stained preparations of RIC revealed the presence of particles with cross-sections of $130 \pm 32 \text{ \AA}$ ($n = 200$). About 3% of the particles had fluid-filled cavities with diameters of $45 \pm 9 \text{ \AA}$ (Fig. 2). It is not known for certain whether these cavities represent the tRNA transport pore. Interestingly, the import substrate tRNA^{Tyr}, but not the nonimported tRNA^{Gln}(CUG), induced a significant increase in the number of cavities (Fig. 2), suggesting these to be RNA-gated channels.

Preliminary mass spectrometric peptide sequencing (data not shown) indicated that the 21-kDa band of RIC contains two major proteins, in keeping with the stoichiometry of two to three copies per RIC (Fig. 1). These include a homologue of assembly subunit 6 (UCR_14kDa) (7) of the ubiquinol-cyto-

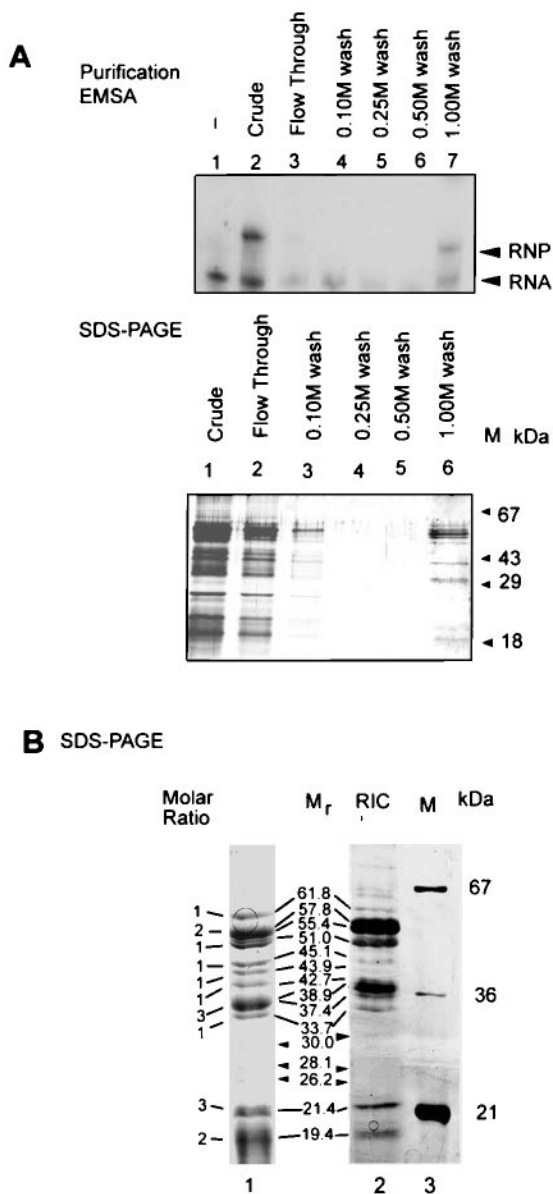


FIG. 1. Purification of RIC from *Leishmania* mitochondria. (A) Upper panel, electrophoretic mobility shift of *Leishmania* tRNA^{Tyr} incubated without protein (lane 1), with crude DOC extract of inner membrane (lane 2), or with the indicated fractions eluting from a D arm affinity column (lanes 3 to 7). Lower panel, SDS-PAGE (silver stain) of the same fractions. M, size markers. (B) SDS-PAGE of RIC. Lanes: 1, Coomassie stain; 2, silver stain; 3, markers. Mole ratios (determined from lane 1) and molecular sizes are shown adjacent to the bands. Substoichiometric bands are indicated by arrowheads.

chrome *c* reductase complex (respiratory complex III) of the inner mitochondrial membrane and an unknown protein with no database matches. The present data are not sufficient to define a precise function of each of these proteins in import but may indicate a close relationship between RIC and the mitochondrial electron transport machinery.

The complex is necessary and sufficient for import. To examine whether the RIC is functional for RNA import, it was incorporated into phospholipid vesicles. RIC-containing vesi-

cles were incubated with radiolabeled tRNA^{Tyr} and then treated with RNase and reisolated. In the presence of ATP, protection of the tRNA from RNase was observed, indicating uptake into the vesicles (Fig. 3A). No protection was observed with RIC-free vesicles. Disruption of the vesicles with DOC after the incubation rendered the RNA nuclease-sensitive, showing that intactness of the membrane is essential for the protection and that the RIC itself does not protect the RNA. The apparent *K_m* for RIC-mediated import of tRNA^{Tyr} into phospholipid vesicles was 1.16 nM (Table 2). tRNA^{Gln}(CUG), which is not imported in vivo (9), was not translocated by RIC (Fig. 3A); the tRNA was recovered intact from the postincubation supernatant (data not shown), arguing against specific degradation of tRNA^{Gln} as the reason for the lack of its import.

Uptake into RIC-reconstituted vesicles was dependent upon ATP; the nonhydrolyzable β-γ imido analogue of ATP was unable to substitute for ATP, indicating the requirement for ATP hydrolysis (Fig. 3A). The apparent *K_m* for ATP was 1.35 mM; this is comparable to the value of 1.52 mM for import into intact mitochondria (2). It has been shown previously that in intact mitochondria or mitoplasts, transfer of tRNA to the matrix requires a transmembrane proton motive force probably generated by the oligomycin-sensitive F₁F₀-ATPase's pumping protons out of the matrix at the expense of ATP hydrolysis (13). In a similar manner, RIC-mediated transfer was sensitive to the protonophore uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, which dissipates proton gradients (Fig. 3A), indicating that both ATP hydrolytic and proton pumping activities are contained within the RIC.

Signal recognition by RIC is sensitive to mutations within the conserved motif. In addition to intact tRNAs, *Leishmania* mitochondria import short oligoribonucleotides containing the import signal YGGYAGAGC in the D arm of tRNA^{Tyr} (2, 3). RIC-dependent transfer of a 27-mer oligonucleotide containing the wild-type sequence was observed (Fig. 3B). The mutation G²²:C in the conserved import motif abolished import, whereas restoration of the loop-closing base pair of the hairpin in the double mutant G²²:C, C¹³:G restored import to about two-thirds of the wild-type level. The mutation A²³:U in the next position of the motif also abolished importability, but restoration of the base pair in A²³:U, U¹²:A failed to regenerate activity. These results indicate that the stability of the hairpin is critical for signal recognition by RIC and, further, that the identity of A²³ is important. The behavior of these

TABLE 1. Enrichment of RIC during purification

Fraction	Protein (mg/ml)	Activity (U/ml) ^a	Total activity (U)	Specific activity (U/mg) ^b	Purification (fold) ^c	Yield (%) ^d
Inner membrane extract	1.2	73	58.4	60	— ^e	100
Affinity 1 M K ⁺ fraction	0.21	510	51	2,380	40	87
Glycerol gradient peak fraction	0.19	700	28	3,684	61	48

^a One unit of activity corresponds to the binding of 1 fmol of tRNA^{Tyr} in the electrophoretic mobility shift assay (Fig. 1).

^b Specific activity is expressed in terms of units per milligram of protein.

^c Increase in specific activity over the crude inner membrane extract.

^d Yield is the percentage of the total activity in the crude extract recovered in each fraction.

^e —, not applicable.

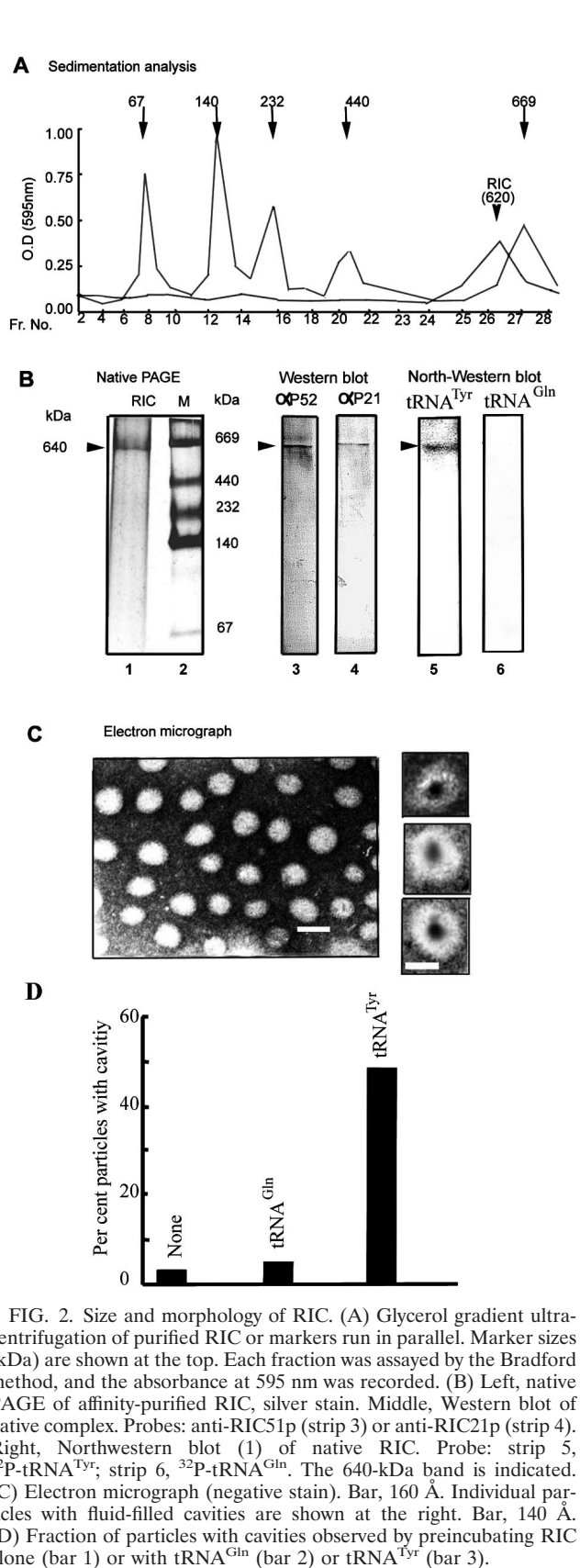


FIG. 2. Size and morphology of RIC. (A) Glycerol gradient ultracentrifugation of purified RIC or markers run in parallel. Marker sizes (kDa) are shown at the top. Each fraction was assayed by the Bradford method, and the absorbance at 595 nm was recorded. (B) Left, native PAGE of affinity-purified RIC, silver stain. Middle, Western blot of native complex. Probes: anti-RIC51p (strip 3) or anti-RIC21p (strip 4). Right, Northwestern blot (1) of native RIC. Probe: strip 5, ³²P-tRNA^{Tyr}; strip 6, ³²P-tRNA^{Gln}. The 640-kDa band is indicated. (C) Electron micrograph (negative stain). Bar, 160 Å. Individual particles with fluid-filled cavities are shown at the right. Bar, 140 Å. (D) Fraction of particles with cavities observed by preincubating RIC alone (bar 1) or with tRNA^{Gln} (bar 2) or tRNA^{Tyr} (bar 3).

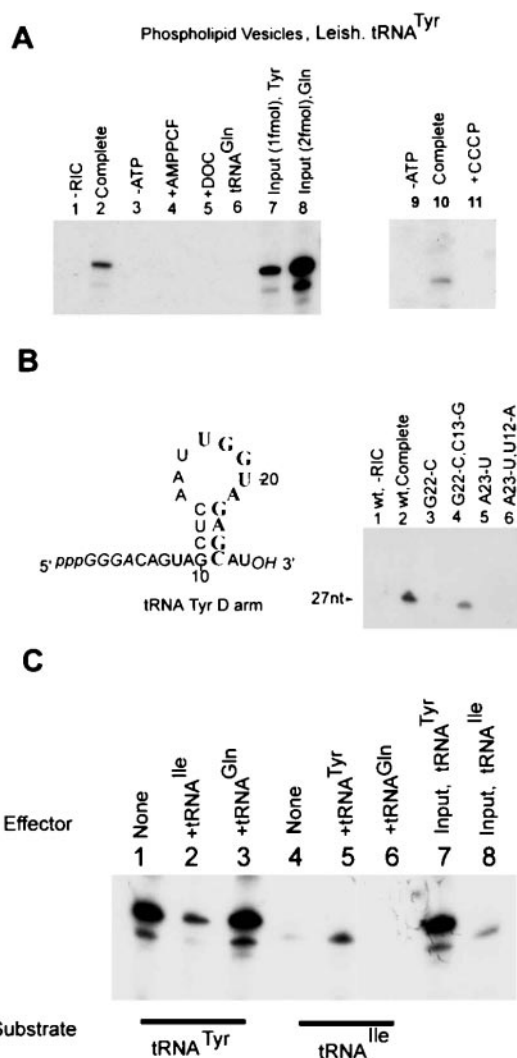


FIG. 3. RIC-mediated import of tRNA into phospholipid vesicles. (A) Import of *Leishmania* tRNA^{Tyr}. Import was assayed by RNase protection. Lane 1, BSA-containing vesicles. Lanes 2 to 6 and 9 to 11, RIC-reconstituted vesicles. The complete system (lanes 2 and 10) contained 100 fmol of tRNA^{Tyr} and 4 mM ATP. Lanes 3 and 9, ATP omitted. Lane 4, ATP replaced by AMPPCP. Lane 5, 0.5% DOC added after import incubation. Lane 6, tRNA^{Gln}(CUG) replacing tRNA^{Tyr}. Lane 11, 50 μM carbonylcyanide *m*-chlorophenylhydrazine added. Lanes 7 and 8, input tRNA^{Tyr} (1 fmol) and tRNA^{Gln} (2 fmol), respectively. (B) Left, structure of tRNA^{Tyr} D arm oligoribonucleotide. Positions are numbered as in the intact tRNA sequence (10). The conserved motif is shown in bold. Right, import of D arm derivatives. Lanes 1 and 2, wild type. Lanes 3 to 6 contained mutants G²²:C; G²²:C, C¹³:G; A²³:U; and A²³:U, U¹²:A, respectively. Lane 1, BSA-containing vesicles. Lanes 2 to 6, RIC-containing vesicles. (C) Interactions between tRNA^{Tyr} and tRNA^{Ile}. RIC-containing vesicles were incubated with high-specific-activity substrate (50 fmol) with or without low-specific-activity effector (5 fmol), and uptake was assayed. Lanes 1 to 3, tRNA^{Tyr} substrate; lanes 4 to 6, tRNA^{Ile} substrate. Lanes 1 and 4, no effector; lane 2, tRNA^{Ile} effector; lanes 3 and 6, tRNA^{Gln}(CUG) effector; lane 5, tRNA^{Tyr} effector. Lanes 7 and 8, input tRNA^{Tyr} and tRNA^{Ile}, respectively (1 fmol).

mutants in the RIC-reconstituted system is identical to that in mitoplasts and different from that in intact mitochondria (2). Thus, RIC retains the RNA recognition specificity of the inner membrane.

TABLE 2. Kinetic characteristics of RIC-mediated import

Experimental variable	Parameter	Value
tRNA ^{Tyr}	K_m^a	1.16 nM
ATP	K_m^a	1.35 mM
tRNA ^{Tyr} (effector) on tRNA ^{Ile} (substrate)	EC_{50}^b	0.033 nM
tRNA ^{Ile} (effector) on tRNA ^{Tyr} (substrate)	IC_{50}^c	0.018 nM

^a Concentration of tRNA^{Tyr} or ATP for half-maximal rate of import into RIC-reconstituted phosphatidylcholine vesicles.

^b Concentration of tRNA^{Tyr} effector required for half-maximal stimulation of binding of tRNA^{Ile} (2 nM).

^c Concentration of tRNA^{Ile} effector for half-maximal inhibition of binding of tRNA^{Tyr} (2 nM).

Allosteric interactions within RIC. A recently described property of inner membrane import is that of cooperative and antagonistic interactions between two types of RNA ligand (3). Type I ligands, of which tRNAs (including tRNA^{Tyr}) containing the conserved D arm domain are an example, can be transferred independently of other ligands, whereas type II ligands (such as tRNA^{Ile}) are dependent on type I ligands for transfer. Moreover, type II ligands inhibit transfer of type I ligands. To examine whether such interactions occur within the isolated complex, RIC-reconstituted phospholipid vesicles were assayed for import of tRNA^{Tyr} in the presence of low concentrations of tRNA^{Ile} and vice versa. Indeed, the uptake of tRNA^{Tyr} was inhibited by tRNA^{Ile}, while tRNA^{Tyr} stimulated uptake of tRNA^{Ile} (Fig. 3C). The 50% effective concentration for tRNA^{Tyr} effector was 0.033 nM, while the 50% inhibitory concentration for tRNA^{Ile} was 0.016 nM (Table 2). Thus, RIC contains receptors for both tRNAs, namely, the primary receptor (that for tRNA^{Tyr}), which binds directly to its ligand, and the secondary receptor (that for tRNA^{Ile}), whose binding to its ligand depends upon conformational changes transmitted within the native complex subsequent to loading of tRNA^{Tyr}.

Regulated interaction of tRNA^{Tyr} with RIC45p. To detect these allosteric interactions at the levels of individual components of RIC, subunit-specific antibodies were employed. A preliminary Northwestern blot analysis of RIC revealed the presence of several tRNA binding proteins. Polyclonal mouse antibodies against these bands were prepared, and their specificity was verified by Western blots (data not shown). Antibody against the 45-kDa subunit (RIC45p) (Fig. 1) inhibited binding as well as import of tRNA^{Tyr} (Fig. 4A). Moreover, in the presence of the antibody, tRNA^{Tyr} was unable to stimulate the import of tRNA^{Ile}, implying either that RIC45p directly binds tRNA^{Tyr} or that it has some other role unrelated to tRNA binding.

To detect a direct interaction between RIC45p and tRNA, a photochemical cross-linking experiment was performed using RNA containing the photoactivable analogue 5-BrU triphosphate (20). Radiolabeled tRNA^{Tyr} containing 5-BrU residues was incubated with mitoplasts and then UV cross-linked to the membrane; after dissociation of protein complexes with detergent, immunoprecipitation was performed with anti-RIC45p antibody. A major cross-linked product, migrating behind free tRNA^{Tyr} and sensitive to protease, was observed (Fig. 4B). This complex was precipitated by anti-RIC45p antibody, but not by normal serum or by antibody against the 21-kDa subunit. Moreover, the yield of the complex was reduced in the presence of tRNA^{Ile} effector in the initial incubation step (Fig.

4B). Thus, RIC45p directly contacts tRNA^{Tyr}, and this binding is inhibited by tRNA^{Ile}, i.e., it has the properties of a type I receptor.

The type I receptor is expected to interact with the corresponding import signal present in the D domain of tRNA^{Tyr}. To examine whether this is the case for RIC45p, a cross-linking experiment was carried out with 5-BrU-labeled D arm oligonucleotide. A specific RNA-protein complex immunoprecipitable with anti-RIC45p antibody was observed (Fig. 4B), showing direct contacts between RIC45p and the D arm signal.

tRNA^{Tyr}-dependent interaction of tRNA^{Ile} with RIC21p. Information was obtained on type II interactions by similar immunochemical experiments. Antibody against the 21-kDa band (RIC21p) had no effect on the binding of tRNA^{Tyr} to the inner membrane and had only a two- to threefold effect on its import (Fig. 4). In contrast, the antibody totally inhibited binding as well as import of tRNA^{Ile} in the presence of tRNA^{Tyr} (Fig. 4). It also antagonized the inhibition of binding and import of tRNA^{Tyr} by tRNA^{Ile}. These results indicate that either RIC21p is the binding site for tRNA^{Ile} or that it somehow relays the conformational changes between type I and type II receptors. With 5-BrU-labeled tRNA^{Ile}, a tRNA-RIC21p complex which migrates slower than the free RNA and which is sensitive to protease (data not shown) was recovered using anti-RIC21p, but not anti-RIC45p, antibody when tRNA^{Ile} was incubated with mitoplasts in the presence of tRNA^{Tyr}, but not in its absence (Fig. 4C). Moreover, the 5-BrU-labeled V-T segment of tRNA^{Ile}, which contains a type II signal, was specifically cross-linked to form a complex immunoprecipitable by anti-RIC21p antibody (Fig. 4D). We conclude that a component of RIC21p has the properties of a type II receptor for tRNA^{Ile}.

DISCUSSION

In this study, progress has been made towards the molecular definition of the inner membrane import machinery of *Leishmania* mitochondria. Affinity chromatography using a type I import signal (i.e., the D arm of tRNA^{Tyr}) was a particularly effective way to separate the functional import complex from the host of respiratory complexes on the inner membrane (Fig. 1). Subsequent resolution of the complex into its constituent polypeptides, followed by a band-by-band immunochemical analysis of the individual components for import-related activities, resulted in the identification of two factors with tRNA receptor function. This biochemical approach, coupled with sequence analysis and genome database searches, should be of general applicability to those systems, e.g., plants and protozoa, in which import mutants are not available.

The complex appears to be composed of roughly spherical particles of uniform size (Fig. 2). A solid protein sphere of 130 Å diameter (as determined by electron microscopy) would have a mass of ~950 kDa, assuming a partial specific volume of 0.73 cm³ g⁻¹ (9). However, the subunit composition, native gel migration, and sedimentation properties support a mass of 620 to 640 kDa (Fig. 1 and 2). This difference can be accounted for by assuming a hollow sphere: if one assumes a cylindrical pore of ~45 Å diameter, the calculated mass of ~750 kDa comes to within 20% of the determined value. The reason for only ~3% of the particles showing fluid-filled cavities may be that the channels are gated by tRNA. This is supported by the obser-

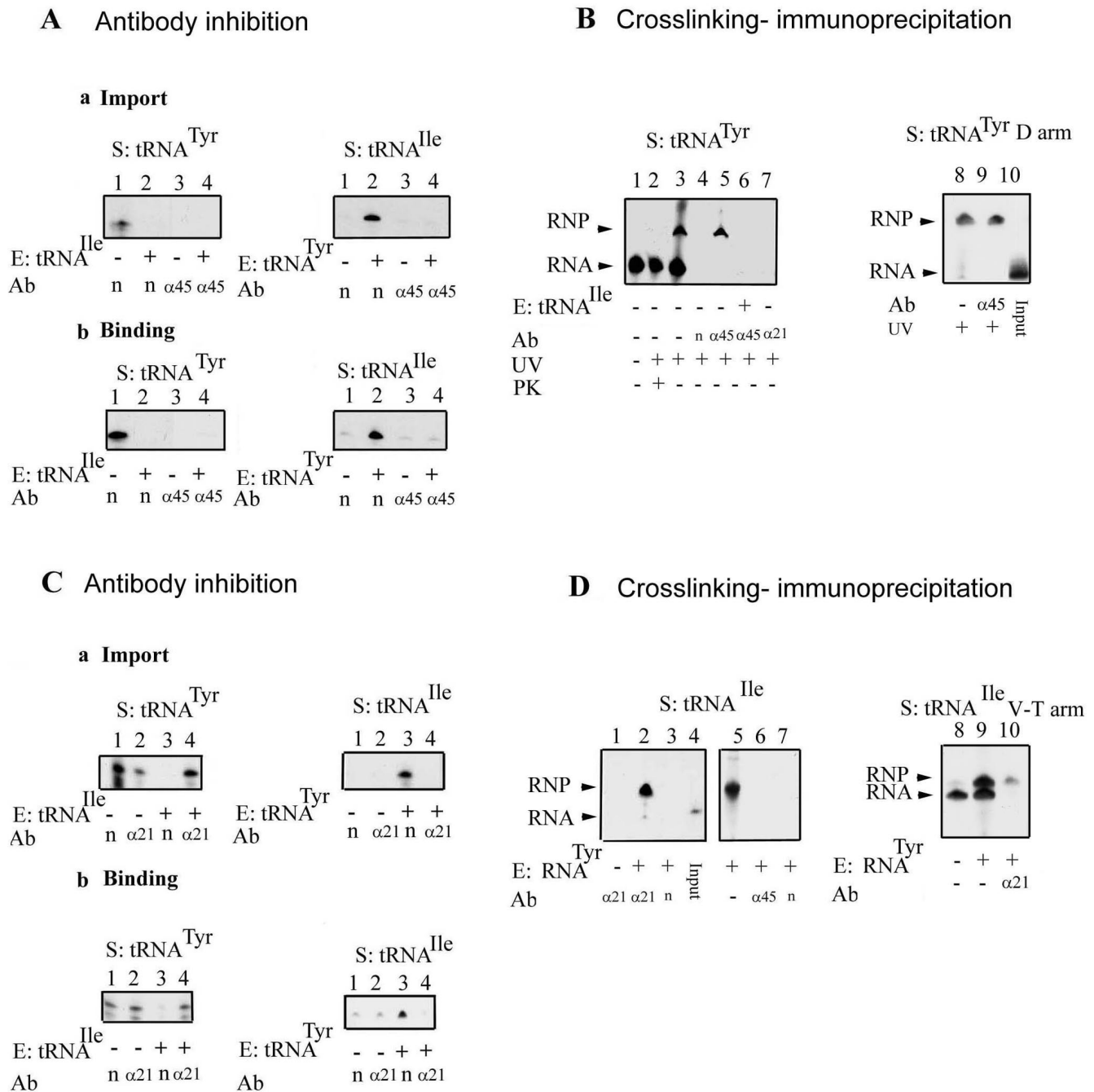


FIG. 4. Role of RIC45p and RIC21p in import. (A) Inhibition by anti-RIC45p antibody. Mitoplasts were incubated with the indicated antiserum (1:50), washed, and assayed for import (a) or binding (b) of the indicated ³²P-labeled tRNA substrate (S) in the presence or absence of the indicated effector (E). Ab, antibody; n, nonimmune serum; α, anti-RIC 45p serum. (B) Immunoprecipitation of cross-linked tRNA. Left, 5-BrU-, ³²P-labeled tRNA^{Tyr} (20 fmol) was incubated with mitoplasts in the presence (lane 6) or absence (lanes 1 to 5 and 7) of low-specific-activity tRNA^{Ile} (2 fmol) under binding conditions. In lanes 1 to 3, the mitoplasts were solubilized and directly loaded; lanes 4 to 7 show immunoprecipitates. RNA-protein complexes were UV cross-linked (except lane 1, a no-UV control) and immunoprecipitated with anti-RIC45p (lanes 5 and 6), anti-RIC21p (lane 7), or nonimmune serum (lane 4). Right, photo-cross-linking of 5-BrU-labeled D arm oligonucleotide (Fig. 3). Lane 10, input RNA. Lane 8, cross-linked product. Lane 9, anti-RIC45p immunoprecipitate. (C) Inhibition by anti-RIC21p antibody. Mitoplasts were incubated with nonimmune or anti-RIC21p antibody and assayed for import (a) or binding (b) of the indicated substrate in the absence or presence of effector. (D) Cross-linking of RIC21p to intact tRNA^{Ile} (left) or tRNA^{Ile} [42-66] (right). Mitoplasts were incubated with the indicated substrates and effectors before UV irradiation. Lane 4, input tRNA^{Ile}. Lane 5, cross-linked product. Lanes 1 to 3, 6, and 7, immunoprecipitates with anti-RIC21p (lanes 1 and 2), nonimmune serum (lanes 3 and 7), or anti-45p antibody (lane 6). Right, tRNA^{Ile} [42-66] was incubated with mitoplasts in the absence (lane 8) or presence (lanes 9 and 10) of tRNA^{Tyr} effector and then irradiated. Lane 10, anti-21p immunoprecipitate.

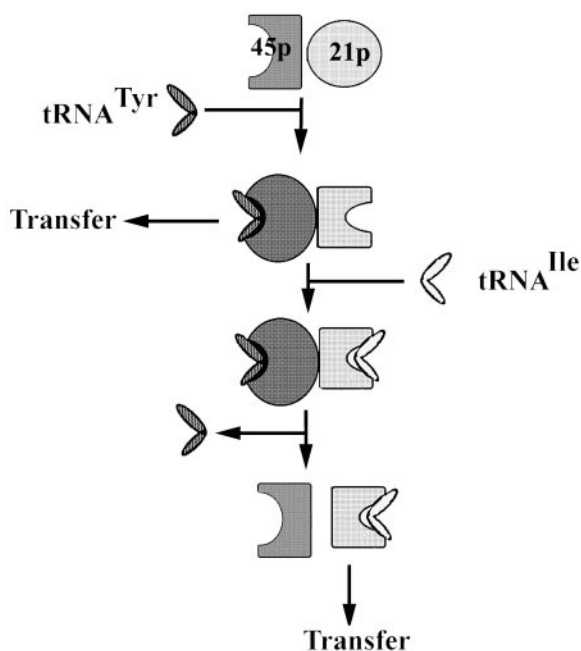


FIG. 5. The “ping-pong” model of allosteric interactions. Heavily and lightly shaded objects represent RIC45p and RIC21p, respectively. Different shapes indicate different conformations. For details, see the text.

vation that in the presence of tRNA^{Tyr}, there is an increase in the fraction of particles showing pores (Fig. 2). Thus, one consequence of the binding of tRNA to its receptor on the RIC would be to induce the opening of the import channel. Assembly of proton translocation channels on the endoplasmic reticulum membrane induced by ribosome “ligand” has been previously observed (6).

The diameter of the putative translocation channel (~45 Å) is considerably larger than the 20-Å protein transport channels on the mitochondrial outer membrane (9) or endoplasmic reticulum (6), which can accommodate unfolded polypeptide chains. The canonical tRNA structure is L shaped, with two 20-Å helical arms at an angle of ~90° and a distance of 80 Å between the two extremities of the L (16). This means that in order to pass through the 45-Å pore, the tRNA structure has to be distorted by reducing or increasing the hinge angle. It may not be a coincidence that both the type I (D arm) and type II (V-T region) are located at or close to the hinge; interaction with the cognate receptors could lead to conformational changes within the tRNA appropriate for translocation.

By antibody inhibition, photochemical cross-linking, and immunoprecipitation, two components of the import complex were identified as proximal contact sites for tRNA^{Tyr} and tRNA^{Ile}, respectively (Fig. 4). The RIC45p-tRNA^{Tyr} complex is inhibited by tRNA^{Ile}, whereas the RIC21p-tRNA^{Ile} complex is stimulated by tRNA^{Tyr}, as expected of type I and type II receptors, respectively. These complexes are specific for their respective tRNAs, i.e., the receptors are distinct. Additionally, blocking of RIC21p by antibody is sufficient to prevent inhibition of tRNA^{Tyr} binding by tRNA^{Ile} (Fig. 4), indicating that a separate tRNA^{Ile} binding protein is not required to negatively regulate the type I tRNA. These findings can be accommo-

dated by a simple two-receptor model (Fig. 5). RIC45p binds tRNA^{Tyr} to form a translocation-competent complex from which the tRNA can be transferred to the import pore. The tRNA^{Tyr}-bound complex contacts RIC21p, activating the tRNA^{Ile} binding site of the latter. Subsequent loading of tRNA^{Ile} on RIC21p results in a second conformational change that is transmitted back to RIC45p, leading to the dissociation of tRNA^{Tyr}, loss of contact between the two receptors, and formation of a translocation-competent RIC21p-tRNA^{Ile} complex. Such feed-forward and feedback interactions involving transient contacts between the respective receptors explain how the type I and type II tRNAs can be transported through the same import pore without directly competing with one another, while maintaining a properly balanced pool within the mitochondrial matrix.

More work is necessary on the identities of these two proteins and on the details of their interactions with tRNAs. Peptide analysis of the 21-kDa component indicated the presence of two or more proteins. It is not obvious which of these is the tRNA^{Ile} binding protein detected by photochemical cross-linking (Fig. 4), as neither contains a known RNA binding domain. The complex III homologue contains an extra N-terminal domain that could be involved in tRNA binding; alternatively, the unknown protein could perform this function. In mitochondria, electron transfer through the respiratory chain results in vectorial proton transport coupled to a number of inner membrane transport processes as well as ATP synthesis. The sensitivity of RIC-mediated tRNA import to uncouplers (Fig. 3) implies a similar electron transfer-coupled proton pumping by the complex and, by inference, the presence of respiratory chain components within RIC. Thus the tRNA import complex may be viewed as a specialized molecular motor combining elements of mitochondrial energy transduction with RNA binding and transport.

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