The α-Subunit of Leishmania F\textsubscript{1} ATP Synthase Hydrolyzes ATP in Presence of tRNA*  

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Import of tRNAs into the mitochondria of the kinetoplastid protozoon Leishmania requires the tRNA-dependent hydrolysis of ATP leading to the generation of membrane potential through the pumping of protons. Subunit RIC1 of the inner membrane RNA import complex is a bi-functional protein that is identical to the α-subunit of F\textsubscript{1}F\textsubscript{0} ATP synthase and specifically binds to a subset (Type I) of importable tRNAs. We show that recombinant, purified RIC1 is a Type I tRNA-dependent ATP hydrolase. The activity was insensitive to oligomycin, sensitive to mutations within the import signal of the tRNA, and required the cooperative interaction between the ATP-binding and C-terminal domains of RIC1. The ATPase activity of the intact complex was inhibited by anti-RIC1 antibody, while knockdown of RIC1 in Leishmania tropica resulted in deficiency of the tRNA-dependent ATPase activity of the mitochondrial inner membrane. Moreover, RIC1 knockdown extracts failed to generate a membrane potential across reconstituted proteoliposomes, as shown by a rhodamine 123 uptake assay, but activity was restored by adding back purified RIC1. These observations identify RIC1 as a novel form of the F\textsubscript{1} ATP synthase α-subunit that acts as the major energy transducer for tRNA import.

A unique feature of kinetoplastid protozoa such as Leishmania and Trypanosoma is that their mitochondrial genome does not contain any tRNA genes; to carry out mitochondrial translation, nucleus-encoded tRNAs are imported from the cytoplasm (1, 2). In other organisms such as higher plants and yeast, variable numbers of tRNAs are imported (reviewed in Ref. 3). Previous in vitro studies using isolated mitochondria from Leishmania, Trypanosoma, yeast, and higher plants showed that tRNA import is dependent on ATP hydrolysis (4–8). In most of these systems, import has been shown to be sensitive to respiratory chain inhibitors and uncouplers, implying the requirement of a trans-membrane proton gradient (7–11). Investigations into the role of ATP have been facilitated by the isolation of a functional multiprotein RNA import complex (RIC) 3 from the inner membrane of Leishmania tropica mitochondria (12). Studies using RIC-reconstituted phospholipid vesicles have shown that tRNA specifically stimulates a RIC-associated ATPase and the generation of an electrochemical potential across the membrane (13).

The molecular identities of the factors mediating these bioenergetic transactions are unknown. Recently, we have identified an essential subunit of the complex, RIC1, to be a bi-functional form of the α-subunit of the F\textsubscript{1}F\textsubscript{0} ATP synthase (14). In common with the ubiquitously conserved F\textsubscript{1}α-subunit, RIC1 has a three-domain structure consisting of an N-terminal β-barrel, a central ATP-binding domain, and a C-terminal domain but differs from the canonical structure in having an extension at the C terminus. Moreover, it specifically binds to a subset of importable tRNAs (Type I) and positively regulates the import of a second subset (Type II) (12, 14). The tRNA binding activity of the protein requires both the ATP-binding domain and the C-terminal domain (14). In the present study, we present evidence demonstrating that RIC1/F\textsubscript{1}α is a novel tRNA-dependent ATPase that is responsible for generating the trans-membrane potential that drives import.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria and Purification of RIC—Mitochondria were prepared from L. tropica strain UR6 and purified by Percoll gradient centrifugation (15). Mitoplasts were obtained by digitonin treatment and inner membrane fractions by repeated freeze-thaw of mitoplasts, as described previously (10). RIC was purified by RNA affinity chromatography from mitochondrial inner membrane fractions (12). The flow-through fraction, depleted of RIC but retaining Complex V, was used as the source of F\textsubscript{1} ATPase.

Cloning, Expression, and Purification of RIC1—RIC1 gene fragments were expressed in E. coli as glutathione S-transferase fusion proteins, excised with thrombin, and gel-eluted in SDS-containing buffer, as described (14). Prior to enzymatic or other assays, the recombinant protein was refolded by diluting 20-fold into TETN250 buffer (12) and incubating for 2 h at 4 °C.

RIC1 Conditional Knockdown Cells—L. tropica strain 13-90, transformed with targeting vector pGET–RIC1(AS), containing the RIC1 gene in antisense orientation, was obtained previously (14). Cultures were induced with 1 μg/ml tetracycline to express anti-RIC1 RNA from a tetracycline repressor-regulated T7 RNA polymerase promoter, resulting in knockdown of RIC1 mRNA and cessation of cell growth within 24 h (14). Mitochondrial extracts from uninduced or 24-h induced cells were prepared with sodium dodecyl maltoside and concentrated by ultrafiltration (14).

Preparation of Proteoliposomes—Empty phosphatidylcholine vesicles were prepared as described (12). Liposomes (50 μg lipid) were reconstituted with RIC (100 ng) or concentrated mitochondrial extract (derived from ~100 μg of mitochondrial mitochondria).
fraction) in the absence or presence of 8 ng of recombinant RIC1, in 20 μl of liposome suspension buffer (12) for 1 h at 4 °C.

Preparation of tRNA—tRNA transcripts were prepared using the corresponding cloned genes as template (12, 14).

ATPase Assay—Recombinant RIC1 fragments, proteoliposomes reconstituted with RIC, or inner membrane fractions were incubated in a 5-μl reaction with 1 mM [γ-32P]ATP (specific activity: 2000 cpm/pmol) in 10 mM Tris-HCl, pH 8.0, 10 mM MgAc2, and 2 mM dithiothreitol. After incubation at 37 °C for 10 min, 1 μl of each reaction was analyzed by polyethyleneimine cellulose TLC as described previously (13).

Rhodamine 123 Uptake Assay—Liposomes (10 μg of lipid) reconstituted with mitochondrial extracts of wild-type or RIC1 knockdown cells were incubated with 0.05% rhodamine 123 in 10 μl of import buffer (15) with 4 mM ATP for 15 min at 37 °C. Wherever indicated, liposomes were preincubated with inhibitors at 4 °C for 15 min. The proteoliposome suspension was mixed with an equal volume of isotonic sucrose (15) containing 20% glycerol and observed and analyzed by confocal microscopy as described (13). The mean fluorescence intensity per particle was calculated as the product of the average intensity in stained particles times the fraction of particles staining above background.

RESULTS

RIC1 Is a tRNA-dependent ATPase—To test the ATPase activity of RIC1, the purified recombinant protein, containing the ATP-binding and C-terminal domains (RIC1 (109–574); see Fig. 1F) was incubated with [γ-32P]ATP in the absence or presence of a tRNA co-factor and hydrolysis monitored by thin layer chromatography. In the presence of three of the six tRNAs tested (tRNA Tyr, tRNA Trp, and tRNA Arg) ATP was hydrolyzed, but the other three tRNAs (tRNA Ile, tRNA Val, tRNA Met-e) were ineffective (Fig. 1A). The former group is classified as Type I and the latter group as Type II (14). Thus, RIC1 is a Type I tRNA-dependent ATPase, in keeping with its known ability to bind this group of tRNAs (12, 14).

Rate curves (Fig. 1B) indicated that the apparent $K_m^{tRNA(Tyr)}$ for ATP hydrolysis by purified RIC1 was 0.25 mM. This is somewhat less than the corresponding value 0.98 nM for ATP hydrolysis by intact RIC (13), implying the presence of inhibitory influences on the RIC1-tRNA interaction within the complex. On the other hand, $K_m^{tRNA(Tyr)}$ of RIC1 was 1.275 mM, similar to the corresponding value of 1.32 mM for the complex (13).

The tRNA Tyr-stimulated ATPase activity of RIC was completely inhibited by preincubating the complex with anti-RIC1 antibody but not with non-immune serum (Fig. 1C), indicating that RIC1 is the sole tRNA-dependent ATP hydrolyzing subunit of the complex.

The D-arm of tRNA Tyr (Fig. 1D) contains an import signal (11, 16) and interacts directly with RIC1 (12). An oligoribonucleotide containing the wild-type D-arm sequence could efficiently stimulate the ATPase activity (Fig. 1E), with an apparent $K_m$ of 0.45 nM, close to the value of 0.25 nM (see above) for the intact tRNA. A G22-C mutation at the loop-closing position (Fig. 1D) resulted in loss of co-factor activity (Fig. 1E), while

FIGURE 1. tRNA-dependent ATPase activity of RIC1. A, ATP hydrolysis by recombinant RIC1-(109–574) (50 fmol) in presence of 1 nM of indicated tRNAs; trY, tRNA Tyr, trW, tRNA Trp, trR, tRNA Arg, trI, tRNA Ile, trV, tRNA Val, trM, tRNA Met-e. B, Rate of ATP hydrolysis by RIC1 (50 fmol) as a function of concentration of ATP (lower) or tRNA Tyr (upper). C, RIC (10 ng)-reconstituted liposomes were preincubated with non-immune serum (lane 3) or anti RIC1 antibody (lane 4), and ATPase activity was assayed in presence (lanes 3 and 4) or absence (lane 2) of tRNA Tyr (1 nM). D, stem-loop structure of the D-arm of tRNA Tyr. The conserved import signal is shown in bold. Mutations at the loop-closing position are indicated. E, ATPase activity of RIC1-(109–574) in the presence of 1 nM of the wild-type (WT) or mutant (G22→C or G22→C, C13→G) D-arm oligonucleotide. F, upper domain structure of RIC1, showing the bacterially expressed fragments. Lower, ATPase activity of indicated RIC1 fragments (50 fmol) in absence or presence of tRNA Tyr, as indicated. G, ATPase activity of RIC1-(109–574) (lanes 2 and 3) or of RIC-depleted F1, enzyme fraction (lanes 4 and 5), in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 50 μM oligomycin. tRNA Tyr (1 nM) was present in reactions 1–3.

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restoration of the base pair in the double mutant G\(^{22}\)→C, C\(^{13}\)→G led to recovery of activity (Fig. 1E). Since the single mutant is defective for import through the inner membrane, while the double mutant is imported (11), this result suggests a direct link between ATP hydrolysis and interaction of RIC1 with the import signal.

The tRNA binding activity of RIC1 involves the cooperative interaction of the ATP-binding and the C-terminal domains (14). When the individual domains were expressed and tested for ATPase activity, both were found to be inactive, whereas in combination, activity was restored (Fig. 1F). The N-terminal domain did not have any effect on the activity.4

Oligomycin is a specific inhibitor of mitochondrial F\(_{1}\)F\(_{0}\) ATP synthase (or ATPase), and \(Leishmania\) mitochondria contain such an oligomycin-sensitive activity (17, 18). The RIC1 ATPase activity was resistant to oligomycin (Fig. 1G). In contrast, a mitochondrial extract depleted of RIC, but retaining the F\(_{1}\)F\(_{0}\) ATPase, had oligomycin-sensitive activity (Fig. 1G). RIC1 is thus distinct from the F\(_{1}\)F\(_{0}\) ATPase.

ATPase Activity in RIC1 Knockdown Cells—Inducible antisense-mediated knockdown of RIC1 in transgenic \(Leishmania\) promastigotes renders the cells non-viable by shutting down mitochondrial translation through impairment of tRNA import into the mitochondria (14). To examine the effect of RIC1 deficiency on the tRNA dependent ATPase, inner mitochondrial membrane fractions were assayed in presence of oligomycin to block the activity of the F\(_{1}\)F\(_{0}\) enzyme. While inner membranes from uninduced \(\text{(i.e.) normal}\) cells had tRNA\(^{\text{Tyr}}\)-dependent ATPase (~50 pmol min\(^{-1}\) μg\(^{-1}\)) no significant activity was detectable in knockdown cells (Fig. 2) indicating that RIC1 is the major, if not the sole, such enzyme in the inner membrane.

RIC1-mediated Generation of Membrane Potential—The membrane potential generated through proton pumping by actively respiring mitochondria drives the uptake of the lipophilic cationic dye rhodamine 123 into the matrix (19). Rhodamine 123 was previously shown to be taken up by isolated \(Leishmania\) mitochondria in presence of ATP (10) and into RIC-reconstituted liposomes in presence of tRNA and ATP (13). To determine the involvement of RIC1 in the generation of membrane potential, liposomes were reconstituted with mitochondrial extracts from RIC1 knockdown cells and assayed for uptake of rhodamine 123. In control experiments, about 81% of the vesicles reconstituted with extracts from un-induced (normal) cells were stained with rhodamine 123 (Fig. 3B). There was virtually no uptake by liposomes reconstituted with RIC1-deficient extracts (Fig. 3C), but uptake occurred if the knockdown extracts were supplemented with purified RIC1 (Fig. 3D). RIC1 could not be replaced by unrelated proteins such as bovine serum albumin, and RIC1 in the absence of mitochondrial extract had no activity.4 Uptake required tRNA and ATP; ATP could not be replaced by AMP-PCP, a non-hydrolysable ana-
logue (Fig. 3, E and F). Moreover, uptake of rhodamine 123 was abolished by the protonophore uncoupler m-chlorocarbonyl-cyanide phenylhydrazone and by oligomycin (Fig. 3F). These results indicate that RIC1, through hydrolysis of ATP, is required for the pumping of protons and generation of membrane potential. Since the ATPase is resistant to oligomycin, whereas rhodamine uptake is sensitive, the generation of the proton gradient must follow ATP hydrolysis and may involve other RIC components.

**DISCUSSION**

Results presented here show that RIC1 is a novel form of the α-subunit of F1F0 ATP synthase having an ATP hydrolase activity that is switched on by tRNA. RIC1/F1α contains the conserved Walker A (P loop) and Walker B motifs that constitute ATP-binding sites in the α- and β-subunits of the F1 sector (20). However, in common with α-subunits from other species, RIC1 lacks a second motif, GERXXE, that is present on β-subunits and contributes to ATP hydrolysis (20). For this reason, the canonical α-subunit has ATP binding but no hydrolytic activity; it is believed to play a regulatory role during ATP synthesis. Thus, the enzymatic activity of RIC1/F1α may be brought about by conformation changes resulting from the cooperative interaction between the ATP-binding domain, the C-terminal domain, and the D domain of tRNA. The situation is reminiscent of the bacterial transcription terminator protein rho, an RNA-dependent ATPase that has the α-type ATP-binding domain (21).

RIC1/F1α is the product of a single gene and is shared between the RNA import complex and respiratory F1F0 ATP synthase (14), implying a bi-functional role in ATP synthesis and tRNA import. The evolution of the new import function in this ancient protein may have been brought about by the incorporation of tRNA-binding sites, ATP hydrolytic activity, and novel protein-protein interaction motifs, enabling the alternative assembly of the same protein into two different macromolecular complexes without disruption of either function.

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