Import of RNA into Leishmania Mitochondria Occurs through Direct Interaction with Membrane-bound Receptors*

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Cytoplasmic tRNAs are imported into the kinetoplast mitochondrion of Leishmania, but the mechanism of import is unknown, particularly whether RNA is transferred as a ribonucleoprotein complex through the protein import pathway or by a distinct receptor-mediated mechanism. Using isolated mitochondria, it was shown that a small, importable RNA, which is structurally homologous to tRNA, binds rapidly, specifically, and with high affinity to the mitochondrial surface in the absence of soluble protein factors to form an import intermediate. Two classes of binding sites of apparent Kd 0.3 and 10 nm, respectively, were distinguished. tRNA from Leishmania, but not yeast, competitively inhibited the binding. Northwestern blot analysis revealed the presence of a 15-kDa RNA binding protein on the mitochondrial surface. Whereas receptor binding was resistant to heparin and KCl, internalization was sensitive to both reagents. These results are consistent with the presence of a direct mechanism of receptor-mediated RNA import on Leishmania mitochondria.

A unique characteristic of the mitochondrion of kinetoplastid protozoa such as Leishmania is the apparent lack of tRNA genes on mitochondrial DNA and the import of nuclear-encoded tRNAs from the cytoplasm (1–8). Import of a few tRNAs into the mitochondria of plants (9) and yeast (10) has also been reported. Recently, in vitro systems were developed for studying this process in yeast (10) and Leishmania (11).

Two alternative models have been considered to explain the transport of a negatively charged RNA molecule across the double mitochondrial membrane. In one scheme, RNA complexed to a cytoplasmic protein is internalized via the well-established protein import pathways (12). Recent studies in yeast suggest that tRNA imports occur as a ribonucleoprotein through the MOM19 protein import receptor (13). For tRNA, the cognate aminoaacetyl tRNA synthetase has been proposed to be the carrier protein (14), but the observation that in Trypanosoma brucei, mutated unspliced tRNA, which cannot be aminoaacetylated, is still imported efficiently (7) would appear to contradict this idea. Alternatively, naked RNA may be directly imported through recognition by membrane-bound RNA binding receptor proteins and specific import channels. Our previous experiments with Leishmania mitochondria showing import of RNA in the absence of cytosolic proteins, in a sequence-specific, saturable, and ATP-dependent manner (11), suggested the second mechanism, but direct evidence was lacking.

In addition to tRNAs and certain small ribosomal RNAs, synthetic transcripts from the 5'-untranslated region of the Leishmania β-tubulin gene are imported in vitro. The anti-sense RNAs are absent in vivo (15) but share purine-rich sequence motifs with the D stem-loop regions of kinetoplastid tRNAs (Ref. 11; see Fig. 1). Moreover, import of antisense RNA is inhibited by tRNA and vice versa (11), and both types of RNA bind to a 15-kDa heat stable protein (15). For quantitative studies of the import mechanism, it is necessary to use a single species of RNA labeled to high specific activity; terminally labeled tRNA mixtures isolated from cellular RNA are therefore unsuitable. Because high specific activity antisense transcripts can be easily synthesized in vitro, we have employed them as a sensitive probe for the import process.

In the present study, we have investigated the presence of import receptors on the mitochondrial surface and examined the relationship between binding and internalization of RNA in order to distinguish between the co-import and direct import models.

MATERIALS AND METHODS

Cell Culture—Promastigotes of Leishmania strain UR6 (a viscero-tropic tropica-like strain) were cultured on solid blood agar as described previously (16).

Isolation of Mitochondria—Mitochondrial vesicles were prepared by hypotonic cell lysis, DNase treatment, and Percoll gradient centrifugation (11, 22). For some experiments, the mitochondrial band was diluted with isotonic sucrose and rerun on a second gradient in order to minimize contamination with cellular debris.

DNA Clones and RNA Probes—Clone pSG3B contains sequences between β-3 and + 25 of the Leishmania β-tubulin gene in transcription vector pSPT19 (15). EcoRI- or HindII-linearized plasmid was transcribed with T7 or SP6 RNA polymerase to generate antisense or sense RNA, respectively (15). Leishmania tRNA was isolated from pol(A)− RNA by denaturing acrylamide gel electrophoresis. Yeast tRNA was purchased from Boehringer Mannheim.

Binding Assays—Unless otherwise specified, binding assays (10 μl) contained 10 mM Tris-HCl, pH 7.5, 0.1 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 1 mM ATP, 6.7 mM creatine phosphate, 60 μg/ml creatine phosphokinase, 3P-labeled RNA (0.1–100 fmol), and 5 μl of mitochondrial suspension (about 20 mg of protein). After incubation at 25°C, vesicles were washed twice with 1 ml of STE-B (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) by low speed centrifugation. Bound RNA was released by deproteinization, purified by ethanol precipitation, and electrophoresed on a denaturing 6% polyacrylamide gel. After autoradiography of the dried gel, the amount of bound RNA was quantified by liquid scintillation counting of the excised band or by scanning the autoradiograms in an Ultrascan laser densitometer (Pharmacia Biotech Inc.).

Import Assays—Internalization of RNA within mitochondria was assayed by RNase protection as described previously (11). Briefly, mitochondria (10 μl in a total volume of 20 μl) were incubated at 25°C with 3P-labeled RNA in binding buffer (see above) lacking KCl (unless otherwise specified) and then treated with a mixture of RNase A (2

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) X51821.

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mg/ml) and T1 (40000 units/ml) for 15 min at 25°C. Mitochondria were reisolated by centrifugation and deproteinized for analysis of RNase-resistant RNA by gel electrophoresis.

Trypsin Treatment of Mitochondria—Mitochondrial vesicles (200 mg of protein) were treated with 10 μg/ml trypsin for 20 min on ice. Trypsin was inactivated with 90 μg/ml soybean trypsin inhibitor for 10 min. Trypsinized vesicles were washed twice with STE-B and either suspended at 4–8 mg/ml in storage buffer (11) or boiled in SDS-PAGE1 sample buffer (17) for electrophoresis.

Northwestern Blot Analysis—Proteins from twice purified mitochondria were resolved by SDS-12% PAGE according to Laemmli (17) and then electroblotted on nitrocellulose (18). To renature proteins in situ, the blot was washed four times with 0.1 M Tris-HCl, pH 7.5, 0.1% Nonidet P-40, each for 30 min at 4°C with gentle agitation. The filter was then blocked with buffer BB(10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol) containing 5% bovine serum albumin and 0.01% Triton X-100, and incubated with 32P-labeled RNA (106 cpm/ml) in BB containing 0.01% Triton X-100 for 4 h at 4°C. After three washes with BB (each for 5 min at 4°C), the filter was autoradiographed.

Succinate Dehydrogenase Assay—Mitochondria were extracted with 0.4% Triton X-100 in STE-B, and the cleared extract was assayed for succinate dehydrogenase essentially as described (19). Reactions (1 ml) contained 50 mM potassium phosphate, pH 7.4, 20 mM sodium succinate, 0.01 mM EDTA, 70 mM 2,6-dichloroindophenol, and 1.6 mM phenazine methosulfate. After the addition of extract the reduction of 2,6-dichloroindophenol was monitored spectrophotometrically at 600 nm. The amount of 2,6-dichloroindophenol reduced was calculated on the basis of its extinction coefficient ε = 21.8 mM−1 cm−1.

RESULTS

Structural Homology between β-Tubulin Antisense RNA and tRNAs—In view of the evidence that synthetic antisense transcripts and Leishmania tRNAs utilize a common import pathway (11), a comparison of their primary and secondary structures was undertaken. As shown in Fig. 1, antisense RNA contains the sequence AUGGCAGAG in the loop of a putative hairpin structure. tRNA\textsuperscript{Thr}(AGU) from \textit{Leishmania tarentolae} contains the identical sequence in the D stem-loop region, whereas tRNA\textsuperscript{Tyr}(GUA) contains the homologous sequence AUUGGUAGAG in the same location (6). Similar structures are conserved in four other \textit{Trypanosoma} tRNA species known to be mitochondrially imported.\textsuperscript{2} In contrast, the D stem-loop of tRNA\textsuperscript{Gln}(CUG), which is not imported (6), contains no such homology (Fig. 1). No significant homology between antisense RNA and any other part of the tRNA cloverleaf was detected. The observed sequence and/or structural similarity explains the action of antisense RNA as a molecular mimic of tRNA for import receptor recognition.

Rapid, Specific Binding of RNA to the Mitochondrial Surface—It was previously observed that import of RNA into isolated Leishmania mitochondria occurs after a pronounced lag (11). The delay could be due to either slow binding of RNA to mitochondrial surface receptors or rapid binding followed by slow internalization. To distinguish between these possibilities, mitochondria were incubated with \textsuperscript{32P}-labeled β-tubulin antisense RNA, the vesicles were washed, and bound RNA was estimated by gel electrophoresis. At 25°C in the absence of ATP, binding was detectable within 5 min, remained stable until 15 min, and then rose steadily (Fig. 2, A and D). In

\textsuperscript{1}The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

\textsuperscript{2}S. Bera and S. Adhya, unpublished data.
presence of ATP, binding was faster and reached a maximum by 15 min (Fig. 2, B and D). The final amounts of complex formed in the two cases were similar (Fig. 2D); therefore, ATP affects the rate but not the yield of the reaction. This rapid binding was in contrast to the slow rate of import, which was detectable only after 30 min (Fig. 2, C and D; see also Ref. 11). Maximum levels of import were reached at 45 min; the subsequent decline in the level of internalized RNA has been occasionally observed (11) and is attributable to intramitochondrial degradation of the latter curve, it was estimated that there are two classes of RNA binding site with apparent dissociation constants (Kd) of 0.3 and 10 nM, present at concentrations ([R]) of 0.4 and 4.7 nM, respectively. Because the protein content of pure vesicles and the extent of contamination of these preparations with other proteins are not precisely known, it is difficult to accurately calculate the number of receptors per mitochondrion, but based on previous observations (5 mg of mitochondrial protein/2 x 10^10 cells containing 1 mitochondrion/cell; Ref. 22), we estimate about 15 molecules of the high affinity receptor and 175 molecules of the low affinity receptor per organelle. These numbers are provisional and subject to future revision.

Receptor Recognition by Homologous tRNA—Because the beta-tubulin antisense transcript used in these assays is absent in vivo (15), the relevance of the data to tRNA import may be questioned. However, competition assays showed that binding of labeled antisense RNA (present at 10 nM) was inhibited by Leishmania tRNA but not by yeast tRNA (Fig. 6). The tRNA concentration for 50% inhibition of binding was 20 nM, similar to that for self-competition by antisense RNA. Thus, the bulk of the homologous tRNA shares a common sequence element for receptor binding that is absent from yeast tRNA.

A 15-kDa RNA Binding Protein on the Mitochondrial Surface—Binding as well as internalization of RNA were sensitive to mild trypsinization of the mitochondrial vesicles (Fig. 7A), intermediate or a nonproductive interaction. Therefore, 15-min complexes were washed free of excess RNA, reincubated in the absence of RNA, and assayed for import by RNase protection. When a complex formed in absence of ATP was incubated without ATP, no import occurred (Fig. 4, lane 2), but the addition of ATP in the second step resulted in import (Fig. 4, lane 3), although the amount internalized was about 2-fold less than in the single-step incubation (Fig. 4, lane 1), possibly due to losses during the washing step. On the other hand, complexes formed in the presence of ATP did not require ATP in the second step for import to occur (Fig. 4, lane 4), and ATP added subsequently had no effect (Fig. 4, lane 5). These results show that at least some of the complexes formed in the absence or the presence of ATP are true intermediates in the sense that the bound RNA can be internalized. However, continued presence of ATP outside the mitochondrion appears to be unnecessary. The internal pool of ATP created by the mitochondrial ATP-ADP carrier may be sufficient to maintain normal levels of import, but other explanations cannot be ruled out at this stage.

Different Classes of RNA Binding Site—To determine the affinity of RNA-receptor interaction, quantitative binding assays were performed at different RNA concentrations (Fig. 5A). Scatchard analysis of the data revealed a nonlinear, biphasic curve (Fig. 5B). From the slopes of the extrapolated linear portions of the latter curve, it was estimated that there are two classes of RNA binding site with apparent dissociation constants (Kd) of 0.3 and 10 nM, present at concentrations ([R]) of 0.4 and 4.7 nM, respectively. Because the protein content of pure vesicles and the extent of contamination of these preparations with other proteins are not precisely known, it is difficult to accurately calculate the number of receptors per mitochondrion, but based on previous observations (5 mg of mitochondrial protein/2 x 10^10 cells containing 1 mitochondrion/cell; Ref. 22), we estimate about 15 molecules of the high affinity receptor and 175 molecules of the low affinity receptor per organelle. These numbers are provisional and subject to future revision.

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indicating the involvement of surface proteins. To identify the putative RNA binding receptor, a Northwestern blot analysis (15) was performed. A Western blot of mitochondrial proteins was probed with labeled antisense RNA. A single band with an apparent molecular mass close to 15 kDa was observed (Fig. 7B, lane 1). No significant band was detected with the corresponding sense strand (control) probe (data not shown). Treatment of mitochondria with trypsin prior to SDS-PAGE resulted in disappearance of this band (Fig. 7B, lane 2). Under the trypsinization conditions used, most of the mitochondrial proteins were unaffected (as determined by Coomassie staining of SDS-PAGE resolved proteins; data not shown), and the activity of the inner mitochondrial membrane marker succinate dehydrogenase was retained (Fig. 7C). These data indicate that the 15-kDa protein is located on the outer membrane with an externally oriented RNA binding domain.

Differential Effects of Salt and Heparin on RNA Binding and Internalization—The response of the binding and internalization steps to mono- and polyvalent ions was investigated. Binding was insensitive to KCl up to 0.1 M; above this concentration, binding was reduced about 2-fold. In contrast, import was inhibited 10-fold above 0.1 M KCl, but the residual activity was resistant to higher concentrations (Fig. 8A). The polyanion heparin was ineffective up to 1 mg/ml in the binding step but quantitatively inhibited internalization at only 20 µg/ml (Fig. 8B). Polynucleotides such as poly(A) and poly(U) had no effect on binding or internalization at 0.1 mg/ml; at 10-fold higher concentration, neither polynucleotide significantly affected binding but completely inhibited import (data not shown). These results are consistent with the occurrence of highly stable and specific, perhaps nonionic, interactions between the receptor and RNA, followed by one or more transfer steps involving more labile, ionic linkages.

DISCUSSION

In this report, evidence was presented for the occurrence of at least one high affinity import receptor for RNA on the surface of Leishmania mitochondria. It was further shown that the binding and internalization steps could be functionally separated on the basis of their biochemical properties. The results point to a unique mechanism for the uptake of cytoplasmic RNAs by the kinetoplast-mitochondrion.

Mitochondrial vesicles bind importable RNAs with high affinity and specificity to form functional import intermediates. The biphasic Scatchard curve (Fig. 5B) suggests the presence of multiple binding proteins or negative cooperativity, i.e. reduced affinity at higher RNA concentrations. Because import is saturated between 1 and 4 nM RNA (11), the high affinity site (Kd = 0.3 nM), is likely to be more relevant to import than the low affinity site (Kd = 10 nM).

A 15-kDa surface protein has many of the expected characteristics of an import receptor. It binds RNA in a sequence-specific manner on Northwestern blots at a probe concentration of only about 0.1 nM (Fig. 7), which indicates a high affinity interaction. We have previously reported the presence of a similar or identical protein in extracts of Leishmania promastigotes (15) or isolated mitochondria (11), which binds to tRNA and other small RNAs that are imported in vitro, to form salt- and heparin-resistant complexes, as observed here with intact mitochondria. Recent immunochemical experiments confirm the role of this protein in RNA import, but whether additional RNA binding proteins are required remains an open question.

Receptor recognition is sequence-specific (Fig. 2) and species-specific (Fig. 6). The fact that the majority of Leishmania and

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Trypanosoma tRNAs are imported argues for the existence of a common primary and/or secondary structure on these RNAs acting as the import signal. Two different heterologous tRNA species from yeast and man, respectively, are apparently imported into Trypanosoma mitochondria (8), a result interpreted to implicate the tRNA structure as a whole as the recognition element. However, in our system, antisense transcripts, which lack a tRNA-like cloverleaf structure, are imported, and total yeast tRNA is ineffective in inhibiting receptor binding (Fig. 6) or import (11). Therefore, it is probable that a short sequence motif shared by all importable RNAs, rather than an elaborate secondary structure, is critical. Sequence analysis pointed to the importance of the nonanucleotide AUGGpyAGAG for receptor recognition (Fig. 1). Our recent structure-function studies support this conclusion.3

It has been suggested that import of tRNA\(^\text{Lys}^1\) into yeast mitochondria occurs through the protein import pathway as a complex with a carrier protein containing an import presequence (13). The properties of the Leishmania system appear to be different. Whereas import of yeast tRNA\(^\text{Lys}^1\) requires a cytosolic protein fraction (10), binding as well as import of RNA into Leishmania mitochondria is direct, and cytosolic protein fractions have no effect.3 The putative RNA import receptor in Leishmania is a 15-kDa protein (Fig. 7), whereas the 19-kDa MOM 19 protein import receptor is implicated in yeast (13). Furthermore, binding of import presequences is transient and salt-sensitive (20, 21), reflecting the presence of salt bridges between the acidic cytosolic domain of the receptor and the positively charged amphiphilic presequence. In marked contrast, binding of RNA to Leishmania receptors is salt- and heparin-resistant, whereas import is sensitive to both reagents (Fig. 8). This indicates that the initial RNA-receptor interaction is stable, followed by one or more transfer steps involving unstable ionic linkages. One possibility is the presence of cationic import channels through which negatively charged RNA

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**Fig. 7.** Identification of an RNA binding protein on the mitochondrial surface. A, effect of trypsin on RNA binding and import. Mitochondria (70 \(\mu\)g of protein) were incubated in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of trypsin, washed, and incubated with antisense RNA (2.5 \(nM\)) for binding (lanes 1 and 2) or import (lanes 3 and 4) assay. B, Northwestern blot analysis. Mitochondria (50 \(\mu\)g of protein) were incubated without (lane 1) or with (lane 2) trypsin and suspended in SDS-PAGE sample buffer. Mitochondrial proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and the blot was probed with labeled antisense RNA (10\(^6\) 

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**Fig. 8.** Effect of KCl and heparin on RNA binding and import. A, Binding (upper panel) or import (lower panel) assays were performed at 2.5 \(nM\) RNA and 0.02, 0.05, 0.1, 0.2, 0.5, and 1 \(mM\) KCl, respectively. B, binding assays were performed at 0, 0.1, 0.2, 0.5, and 1 \(mg/ml\) of heparin, respectively (upper panel). Import assays were carried out at 0, 0.01, 0.02, 0.05 0.1, and 0.2 \(mg/ml\) heparin, respectively (lower panel).
is extruded by transient salt bridging. Further studies on the
structure of the import receptor as well as that of the RNA
import signal will shed more light on this important question.

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