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Role of an RNA-binding Protein in Import of tRNA into *Leishmania* Mitochondria*

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Nuclear-encoded cytoplasmic tRNAs are imported into the mitochondria of kinetoplastid protozoa by an unknown mechanism. In a *Leishmania in organello* system, ATP-dependent import of a cloned, unspliced $tRNA^{Tyr}$(GUA) transcript was demonstrated by protection from ribonuclease, whereas import of a $tRNA^{Gln}$(CUG) transcript was much less efficient. Specific binding of $tRNA^{Tyr}$ to two mitochondrial surface proteins of 15 and 22 kilodaltons was observed. Tubulin antisense-binding protein (TAB), the 15-kilodaton species, was purified to apparent homogeneity by RNA affinity chromatography. TAB forms stable complexes with the D stem-loop region of $tRNA^{Tyr}$. Immunocytochemical and cell fractionation experiments, combined with limited proteolysis, suggested the association of TAB with the outer mitochondrial membrane. Importantly, anti-TAB antibody specifically inhibited binding as well as import of $tRNA^{Tyr}$ and of a synthetic structural homolog. These results support the role of TAB as a membrane-bound receptor or carrier for RNA import into *Leishmania* mitochondria.

Transport of macromolecules across biological membranes is currently a topic of considerable research interest. A great deal is known about protein and ribonucleoprotein transport across the nuclear, mitochondrial, endoplasmic reticulum, and plasma membranes (for recent reviews, see Refs. 1 and 2). Evidence has also accumulated for transport of small RNAs from the cytoplasm to mitochondria, but, until recently, little was known about the mechanism of this process for lack of suitable *in vitro* systems.

In yeast, a single nuclear-encoded tRNA species, $tRNA_1^{Lys}$, is mitochondrially imported (3). tRNA import has also been observed in plant (4) and *Tetrahymena* (5) mitochondria. The most extensive use of a tRNA import mechanism occurs in the mitochondria of kinetoplastid protozoa including *Trypanosoma* and *Leishmania* (6–12). Import of a large number of tRNAs is necessitated by the apparent lack of tRNA genes in the mitochondrial DNA of these organisms. To date, 9 different nuclear-coded tRNA species, including $tRNA^{Tyr}$(GUA), $tRNA^{Lys}$(UUU), and $tRNA^{Thr}$(CGU), are known to be abundant in mitochondria, $tRNA^{Leu}$(CAG) and $tRNA^{Arg}$(UCG) are present at low concentrations, while $tRNA^{Gln}$(CUG) is undetectable in the organelle.

The development of *in vitro* RNA import systems in yeast (3) and *Leishmania* (13) has allowed the import mechanism to be explored. Using the latter system, we showed that small synthetic transcripts (antisense RNA from the 5′-untranslated region of the β-tubulin gene) structurally homologous to tRNA are efficiently imported (13). The antisense transcript contains the sequence AUGGCAGAG in a putative hairpin-loop which is homologous or identical to a part of the D stem-loop of a number of imported tRNAs (14). It has been recently shown that the D stem-loop plays an important part in import *in vivo* (12). We previously observed that total *Leishmania* tRNA enters isolated mitochondria to become labeled with UTP at the 3′ termini by terminal uridylyl transferase in the matrix (13). This reaction is specifically inhibited by β-tubulin antisense transcripts, indicating the use of a common pathway by the two RNAs (13). In the present study we have used cloned tRNA species to directly examine the import process.

Import is ATP-dependent, sequence-specific and saturable by RNA, indicating a receptor-mediated process. The presence of one or more high-affinity RNA-binding sites on the mitochondrial surface was subsequently demonstrated (14), but the identity of the receptor remains unknown. A 15-kDa heat-stable protein which specifically binds to β-tubulin antisense RNA (the tubulin antisense-binding protein, or TAB)[1] was previously detected in crude extracts of *Leishmania* promastigotes (15). A similar or identical protein is also present on the mitochondrial surface (14). In the present study we report the purification of TAB protein and immunochemical experiments to investigate the role of TAB in mitochondrial tRNA import.

MATERIALS AND METHODS

Cell Culture and Preparation of Mitochondria—Leishmania strain UR6, a *tropica*-like strain, was cultured on solid blood agar (16) or in medium 199 containing 10% fetal calf serum. Mitochondria were isolated by Percoll gradient centrifugation following the method of Harris *et al.* (13, 17). Mitochondria (100–200 μg of protein) were treated with trypsin (10 μg/ml) for 20 min on ice, trypsin was inactivated with 90 μg/ml soybean trypsin inhibitor and 0.5 mM phenylmethylsulfonyl fluoride for 10 min, and the vesicles were washed twice with STE-B (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) before use.

Enzyme Assays—Mitochondrial proteins were solubilized with 0.3–0.4% Triton X-100 in STE-B. The soluble lysate was spectrophotometrically assayed for succinate dehydrogenase by the reduction of 2,6-dichlorophenolindophenol as described previously (14). Kynurenine hydroxylase was assayed by the method of Hayaishi (18). Reactions (1 ml) containing 0.1 M potassium phosphate, pH 8.0, 0.2 mM NADPH, 10

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X51821 and X98595.

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[1] The abbreviations used are: TAB, tubulin antisense-binding protein; NADP, nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

mM KCl and mitochondrial extract were initiated by addition of L-kynurenine to 0.1 mM. The decrease in absorbance at 340 nm relative to a control lacking kynurenine was measured over a period of 15 min. Conversion of 0.1 μmol of kynurenine to 3-hydroxykynurenine with simultaneous oxidation of NADPH results in a decrease in optical density of 0.108 at 340 nm (based on the extinction coefficients of 3290, 2200, and 6220 for kynurenine, 3-hydroxykynurenine, and NADPH, respectively).

DNA Clones and RNA Probes—Clone pSG3B contains sequences between +25 and −53 of the *Leishmania* β-tubulin gene in vector pSPT19 (15). The homologous $tRNA^{Tyr}$(GUA) gene was isolated by polymerase chain reaction amplification (19) of *Leishmania* strain UR6 genomic DNA (0.5 μg in a 25-μl volume) using the primers O-34: GGGGTACCTTCTGTAGCTCAATTGGT corresponding to the 5′-terminal 20 nucleotides of mature $tRNA^{Tyr}$(GUA) of *Leishmania tarentolae* (8) plus *Kpn*I linker, and O-35: GGAATTCTCCTTCCGGCCGGAATCG complementary to the 3′-terminal 18 nucleotide plus *Eco*RI linker. The gel-purified polymerase chain reaction product was digested with *Eco*RI and *Kpn*I and inserted into the polylinker of pBluescript SK(+) (Stratagene) to yield pSKB-1. The gene for $tRNA^{Gln}$(CUG) was similarly cloned as recombinant pSKB-2 using polymerase chain reaction primers O-36: CCGGTACCGCTCCTATAGTGTAGCGGTT and O-37: GGAATTCACTCCTACCTGGACTCGAAC corresponding to the 5′- and 3′-terminal sequences of *L. tarentolae* $tRNA^{Gln}$(CUG) (8). Plasmid pSKB-1(Δ-1), containing nucleotides 1–36 of the $tRNA^{Tyr}$(GUA) gene, was obtained by digesting pSKB-1 with *Bam*HI, limited exonuclease III treatment followed by S1 nuclease and T4 DNA polymerase, insertion of a *Bam*HI linker adapter, and recircularization with T4 DNA ligase. The sequences of both strands of each clone were determined by dideoxy chain termination. pSG3B(poly(A)), the template for synthesis of poly(A)-tailed antisense RNA, was derived from pSG3B by addition of the $(dA:dT)_{30}$ sequence from pSP64(poly(A)) (Promega). Standard procedures were followed for recombinant DNA manipulations (20). Runoff transcripts from *Eco*RI-linearized (or *Bam*HI-linearized in case of pSKB-1(Δ-1)) plasmids were synthesized using T7 RNA polymerase and [α-^{32}P]UTP, as described previously (15). Commercial yeast tRNA (Boehringer Mannheim) and gel-purified *Leishmania* tRNA were used where indicated.

Import and Binding Assays—^{32}P-Labeled RNA was incubated with isolated mitochondria and import was monitored by protection from RNase (13). Briefly, reactions (20 μl) contained 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP, 6.7 mM creatine phosphate, 60 μg/ml creatine phosphokinase, ^{32}P-labeled RNA, and 10 μl (80 μg of protein) of mitochondria in storage buffer (0.25 M sucrose, 50% glycerol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). After incubation for 45 min at 25 °C, 0.1 mg/ml RNase A and 2000 units/ml RNase T1 were added and incubated for 15 min at 25 °C. STE-B buffer (500 μl) was added, mitochondria were reisolated by centrifugation, resuspended in 20 μl of STE-B, digested with 1 mg/ml proteinase K for 5 min at 25 °C, then SDS was added to 0.5% and incubation continued for 10 min more. After phenol extraction and ethanol precipitation, RNA was resolved by 8 M urea-6% PAGE. Binding of RNA to the mitochondrial surface was assayed in 10- or 20-μl reactions containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP (as indicated), 6.7 mM creatine phosphate, 60 μg/ml creatine phosphokinase, 0.1 M KCl, ^{32}P-labeled RNA and mitochondria as indicated. After incubation at 25 °C for the specified times, vesicles were washed twice with STE-B (1 ml each), deproteinized, and bound RNA analyzed by denaturing gel electrophoresis.

Northwestern Blot Analysis—Proteins resolved by SDS-PAGE (21) were transferred to nitrocellulose, renatured *in situ*, and probed with ^{32}P-labeled RNA as described (15).

Purification of TAB—Promastigotes of *Leishmania* strain UR6 were lysed with Triton X-100, and dialyzed S-100 extracts were prepared as described previously (15), except that two successive extractions were carried out with 0.1% Triton X-100 buffer and the extracts pooled before dialysis. The extract was heated at 55 °C for 7 min, cooled on ice, denatured proteins removed by centrifugation, and the supernatant immediately subjected to RNA affinity chromatography.

To prepare the affinity column, poly(A)-tailed pSG3B RNA (50–100 μg) in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA was slowly passed through a 1-ml oligo(dT)-cellulose column under gravity, the flow-through was collected and passed a second time through the column. The matrix, containing immobilized RNA, was washed twice with 20 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 1 mM EDTA and equilibriated with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol (Buffer DB) containing 0.1 M NaCl. Subsequent operations were carried out at 4 °C.

The heat-treated S-100 (about 1 mg of protein in buffer DB containing 0.1 M NaCl) was loaded on the RNA affinity column (1 ml) and passed twice at 0.25 ml/min. The column was washed successively with (i) 3 ml of DB containing 0.1 M NaCl, (ii) 5 ml of DB containing 0.25 M NaCl and 100 μg/ml heparin, (iii) 5 ml of DB containing 0.25 M NaCl only, and (iv) 3 ml of DB containing 1 M NaCl. Fractions (0.5 ml) were collected at each step and 1 μl was assayed for TAB activity by gel retardation of ^{32}P-labeled β-tubulin antisense RNA (2 fmol), as described (15). In some cases, the 1 M NaCl eluate was diluted to 0.25 M in NaCl with DB and rechromatographed on the affinity column. Fractions of the 1 M NaCl eluate containing TAB activity were pooled, dialyzed against DB containing 5% glycerol for 2 h with 2 changes, concentrated about 10-fold by dialysis against dry Sephadex, and stored at −70 °C. Fractions were analyzed by silver staining (22) of proteins resolved by SDS-PAGE. For peptide sequencing, affinity-purified TAB was run on SDS-PAGE and the 15-kDa subunit electrotransferred to a polyvinylidene difluoride membrane. Automated amino acid composition and N-terminal sequence analysis were performed at the UCLA Protein Microsequencing Facility, Los Angeles, CA.

Western Blotting—Antibody against the gel-purified 15-kDa subunit was raised in rabbits by 3 injections of about 100 μg each at 10-day intervals. Antibody titers were determined by enzyme-linked immunosorbent assay. IgG was purified from serum by protein A-agarose chromatography (22). Protein A (Sigma) was labeled with $Na^{125}I$ in the presence of sodium metabisulfite (22). Western blots on polyvinylidene difluoride or nitrocellulose membranes were blocked with TBS containing 5% bovine serum albumin for 1 h at room temperature, then incubated with 10 μg/ml IgG or 1:100 dilution of antiserum in blocking buffer for 18 h at 4 °C, washed 3 times with TBS, then treated with 10^5-10^6 cpm/ml ^{125}I-labeled protein A for 1 h. After 3 washes with TBS the blots were autoradiographed.

Immunocytochemistry—Promastigotes were washed with PBS, suspended at about 10^6 organisms/ml in PBS, and applied to poly-L-lysine-coated coverslips. After immobilization for 30 min at room temperature, the coverslips were washed with 3–4 times with PBS. Cells were fixed with ice-cold methanol/acetone (1:1) for 5 min, washed with PBS, then blocking solution (PBS containing 10% goat serum) was added for 30 min. The blocked coverslips were incubated with antiserum (1:100 dilution in blocking solution) for 2 h at 4 °C, washed 3–4 times with PBS, then incubated with FITC-conjugated goat anti-rabbit IgG (1:100) for 1 h at room temperature. After a further 3–4 washes with PBS, the coverslips were mounted on PBS containing 10% glycerol and 0.1 μg/ml ethidium bromide. Stained cells were examined at magnification × 1000 under ultraviolet illumination using either FITC or rhodamine filter in a Leitz Diaplan microscope.

Immunodecoration and Antibody Inhibition—Isolated mitochondria were blocked with buffer STE-B containing 4 mg/ml bovine serum albumin (or 10% goat serum for immunofluorescence) for 30 min on ice, then incubated with 20 μg/ml IgG for 1 h at 4 °C, and washed twice with cold STE-B by low-speed centrifugation. For immunodecoration, IgG-treated mitochondria were incubated with 5×10^6 cpm/ml ^{125}I-labeled protein A for 30 min, washed again, and counted in a γ-counter. For fluorescence microscopy, the mitochondria were incubated with FITC-conjugated anti-IgG (1:100) for 1 h on ice, washed, and applied on poly-L-lysine-coated coverslips for 30 min. Adhered mitochondria were washed and examined as for whole cells. Antibody inhibition experiments were performed with the IgG-treated mitochondria which had been resuspended in mitochondrial storage buffer (13) at 8 mg/ml protein.

RESULTS

Import of $tRNA^{Tyr}$(GUA) into Isolated Mitochondria—To study the import of specific tRNAs *in vitro*, the gene for *Leishmania* $tRNA^{Tyr}$(GUA), which is known to be imported (8), was cloned into a transcription vector and T7 RNA polymerase runoffs were employed in import assays. The sequence of the RNA includes an 11-nucleotide intron (positions 38–48) and is identical to the corresponding tRNA species from *L. tarentolae* (8) except for a U to C base change at position 42. Two differences (A to U at position 41 and U to C at position 42) from *T. brucei* $tRNA^{Tyr}$ (7) were also observed.

When the ^{32}P-labeled $tRNA^{Tyr}$(GUA) transcript was incubated with isolated mitochondria in the presence of ATP, import of the RNA was observed by protection from added RNase (Fig. 1, *lane 2*). As a positive control, antisense RNA was

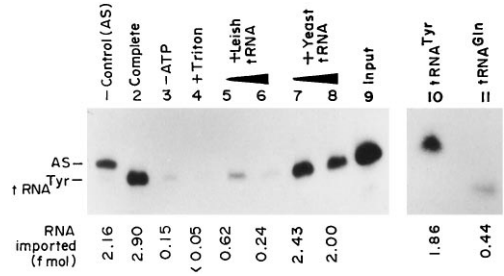

FIG. 1. **Import of tRNA^{Tyr}(GUA) transcript into isolated *Leishmania* mitochondria.** Mitochondria (80 μg of protein) were incubated with 100 fmol of ^{32}P-labeled β-tubulin antisense RNA (*lane 1*), tRNA^{Tyr}(GUA) transcript (*lanes 2–8* and *10*), or tRNA^{Gln}(CUG) transcript (*lane 11*) in a 20-μl reaction volume at 25 °C for 45 min and import assayed by protection from RNase. *Lanes 1, 2, 10,* and *11*, complete system. *Lane 3*, ATP omitted. *Lane 4*, 0.5% Triton X-100 added after incubation but before RNase. *Lanes 5* and *6*, unlabeled total *Leishmania* tRNA (10 and 20 pmol, respectively) added. *Lanes 7* and *8*, unlabeled yeast tRNA (10 and 20 pmol, respectively) added. *Lane 9*, input tRNA^{Tyr} transcript (10 fmol). Import was quantified by liquid scintillation counting of excised gel bands. The lengths of antisense RNA (*AS*), tRNA^{Tyr} and tRNA^{Gln} transcripts are 110, 103, and 91 nucleotides, respectively, and their specific activities were 6.9×10^6, 5.4×10^6, and 4.3×10^6 cpm/pmol, respectively.

imported with comparable efficiency (Fig. 1, *lane 1*). Import of tRNA^{Tyr} transcript was reduced by 95% in the absence of ATP (Fig. 1, *lane 3*). Disruption of the vesicles with Triton X-100 after incubation rendered the RNA totally nuclease-sensitive (Fig. 1, *lane 4*), as expected. Import of labeled tRNA^{Tyr} was inhibited more than 90% by excess *Leishmania* tRNA, but yeast tRNA competed inefficiently (Fig. 1, *lanes 5–8*), implying recognition of specific import signals on cognate tRNAs.

In *L. tarentolae*, tRNA^{Gln}(CUG) is predominantly cytosolic, implying that mitochondrial import of this molecule is inefficient or absent (8). When a cloned tRNA^{Gln}(CUG) transcript was tested *in vitro*, its uptake was observed to be significantly less efficient (by a factor of 4) than that of tRNA^{Tyr} (Fig. 1, *lanes 10* and *11*). Moreover, titration experiments revealed that this low level of uptake was maintained between 1.25 and 5 nM RNA, whereas that of tRNA^{Tyr} increased within this range.[2] The observed qualitative and quantitative similarities of import of tRNA^{Tyr} (Fig. 1) and antisense RNA (11) implicate a common transport mechanism for these two RNAs.

Binding of tRNA^{Tyr}(GUA) to the Mitochondrial Surface—The import process may be subdivided into two steps: binding of RNA to mitochondrial surface receptors, followed by transfer through import channels (14). When tRNA^{Tyr} transcript was incubated briefly with mitochondria, it became specifically associated with the organelle (Fig. 2*A, lanes 1* and *2*), apparently with the outer surface, as judged by the RNase sensitivity of the complex (Fig. 2*A, lane 3*; the small amount of resistant RNA may be due to low-level import within 15 min of incubation). In contrast to the import reaction, complex formation at 15 min was not dependent on ATP, but ATP stimulated binding about 2-fold (Fig. 2*A, lane 4*). Moreover, binding of tRNA^{Tyr} transcript was not significantly inhibited by a 100–200-fold excess of heterologous tRNA (Fig. 2*A, lane 5*), as expected for a specific, high-affinity interaction.

In the presence of ATP, the initial rate of complex formation was stimulated but the final yield at 60 min was not appreciably altered (Fig. 2*C*). Similar rate effects of ATP were observed previously with antisense RNA (13).

To identify the mitochondrial protein(s) to which tRNA binds, Western blots of mitochondrial proteins were probed with ^{32}P-labeled tRNA^{Tyr}(GUA) transcript. This experiment

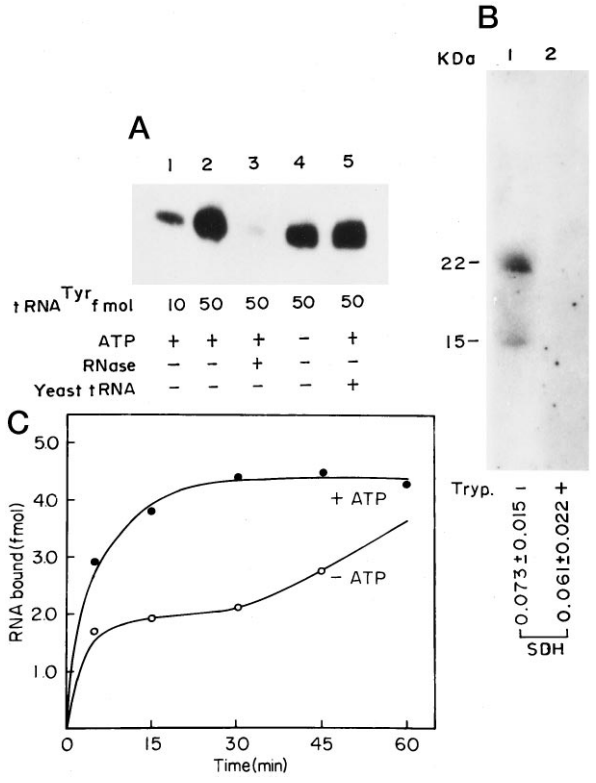

FIG. 2. **Binding of tRNA^{Tyr} transcript to the mitochondrial surface.** *A*, ^{32}P-labeled tRNA^{Tyr} was incubated with mitochondria (80 μg of protein) in 20 μl for 15 min at 25 °C. Vesicles were washed, deproteinized, and bound RNA resolved by gel electrophoresis. *Reactions 1* and *2–5* contained 10 and 50 fmol of tRNA^{Tyr}, respectively. *Lanes 1* and *2,* complete system. *Lane 3*, RNase added after incubation. *Lane 4,* ATP omitted. *Lane 5*, yeast tRNA (10 pmol) added. *B*, Northwestern blot analysis. Mitochondrial proteins (50 μg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with ^{32}P-labeled tRNA^{Tyr}. *Lane 1,* intact mitochondria. *Lane 2*, trypsinized mitochondria. The activity (in nanomoles of dichlorophenolindophenol reduced/min/μg of protein) of succinate dehydrogenase (*SDH*) in each preparation (average of four independent determinations) is shown at the bottom. *C*, time course of tRNA binding. Binding reactions (10 μl) containing mitochondria (35 μg) and ^{32}P-labeled tRNA^{Tyr} (50 fmol) were incubated for 5–60 min at 25 °C in the absence (○) or presence (●) of 1 mM ATP and the bound RNA quantified by gel electrophoresis. Effects of ATP were monitored in reactions lacking the ATP-regenerating system (creatine phosphate and creatine phosphokinase).

showed the presence of two major tRNA-binding polypeptides of 22 and 15 kDa, respectively (Fig. 2*B, lane 1*). Mild trypsinization of mitochondria resulted in disappearance of both bands (Fig. 2*B, lane 2*). Under these proteolytic conditions, inner mitochondrial membrane markers such as succinate dehydrogenase were not significantly affected (Fig. 2*B*). This indicates that both of these RNA-binding proteins are localized on the outer mitochondrial membrane.

Purification of TAB Protein—The heat-stable 15-kDa TAB in promastigote extracts binds to β-tubulin antisense RNA as well as tRNA (15); the same or similar factor is also present on the mitochondrial surface (14). For further studies, TAB was purified from postribosomal supernatants of detergent extracts by a two-step protocol. After heat treatment to remove bulk protein, the extract was passed through an affinity matrix consisting of antisense RNA immobilized on oligo(dT)-cellulose via a synthetic poly(A) tail (Fig. 3*A*). TAB was retained by this column, and after several washes to remove nonspecifically adsorbed protein, eluted with 1 M NaCl (Fig. 3*B*). SDS-PAGE analysis demonstrated the presence of the 15-kDa TAB in the 1 M NaCl fraction at greater than 95% purity (Fig. 3*C*). The RNA binding property of this species was confirmed by 1) rebinding to the

[2] S. Adhya, T. Ghosh, A. Das, and S. Mahapatra, unpublished data.

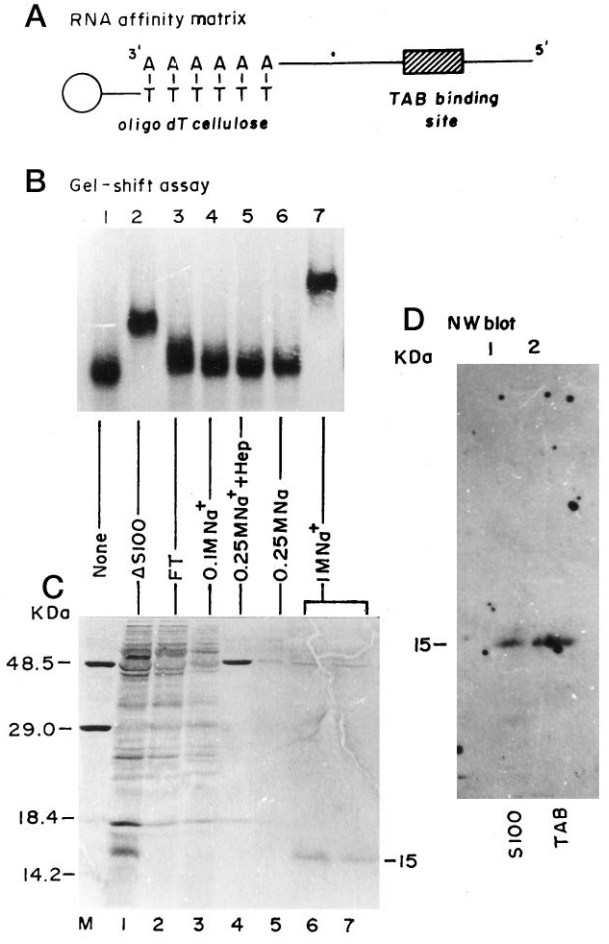

FIG. 3. **Purification of TAB by affinity chromatography.** *A*, design of affinity matrix. β-Tubulin antisense RNA containing TAB-binding sites was hydrogen-bonded to cellulose-linked oligo(dT) via a 3′-poly(A) tail. *B*, activity of column fractions. ^{32}P-Labeled antisense RNA (2 fmol) was incubated with the following fractions (1 μl each) and complexes analyzed by gel-shift assay: *lane 1*, none; *lane 2*, 55 °C-treated S-100; *lane 3*, flow-through; *lane 4*, 0.1 M NaCl wash; *lane 5*, 0.25 M NaCl + 100 μg/ml heparin wash; *lane 6*, 0.25 M NaCl wash; *lane 7*, 1 M NaCl eluate. *C*, silver-stained proteins in column fractions resolved by SDS-12% PAGE. Samples in *lanes 1–5* were identical to those in *lanes 2–6*, respectively, of *panel B; lanes 6* and *7* contained Sephadex-concentrated 1 M NaCl eluate (approximately 600 ng). *D*, Northwestern blot probed with ^{32}P-labeled antisense RNA. *Lane 1*, 55 °C-treated S-100 (25 μg); *lane 2*, affinity-pure TAB (approximately 3 μg).

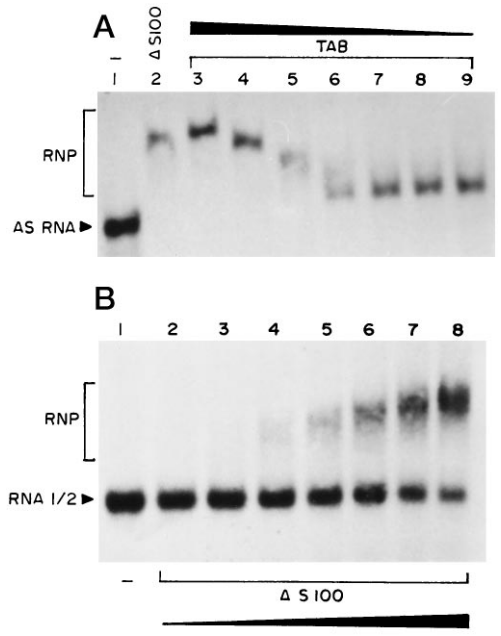

FIG. 4. **Interaction of TAB with RNA in solution.** Binding assays (10 μl) contained 10 mM HCl, pH 7.5, 5 mM $MgAc_2$, 2 mM dithiothreitol, 5 mg/ml heparin, and ^{32}P-labeled RNA and extract or purified factor as indicated. After a 30-min incubation on ice, complexes were analyzed on native gels as described (15). *A*, β-tubulin antisense RNA (2 fmol) incubated without protein (*lane 1*) or with 1.5 μg of heat-treated S-100 extract (*lane 2*), or with 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 ng of affinity-purified TAB (*lanes 3–9,* respectively). *B*, half-$tRNA^{Tyr}$ (2 fmol) was incubated with none (*lane 1*) or with 17, 34, 68, 137, 275, 550, and 1100 ng of heat-treated S-100 fraction of TAB (*lanes 2–8*, respectively). The length of the half-tRNA is 68 nucleotides, and its specific activity, 2.3×10^6cpm/pmol.

matrix[2] and 2) Northwestern blot analysis using ^{32}P-labeled antisense RNA probe (Fig. 3*D*).

Purified TAB was subjected to Sephacryl S-200 chromatography. RNA binding activity eluted at a position corresponding to a native molecular mass of 30 kDa.[2] Thus, in solution, TAB exists primarily as a dimer of the 15-kDa subunit.

The purified 15-kDa subunit contains 20% acidic, 17% basic, and 15% hydrophobic amino acid residues, with the N-terminal sequence ADTRSYYNGG. The absence of an N-terminal methionine indicates that it is a processed protein.

Interaction of TAB with RNA—Binding of solubilized TAB with RNA was monitored by the gel retardation assay (15). The reaction was optimized with respect to various parameters. Binding occurs rapidly (within 5 min) at 4 °C in the presence of 0.1–10 nM RNA and is not affected by monovalent cations (1 M), heparin (5 mg/ml), or synthetic polynucleotides (100 μg/ml). ATP (1 mM) or EDTA (1 mM) had no effect on the mobility or yield of the complex.[2]

When antisense RNA was titrated with affinity-purified TAB, stepwise formation of 3 discrete complexes of decreasing mobility were observed (Fig. 4*A*). Similar results were previously obtained with various deletion derivatives of this RNA, tRNA as well as small ribosomal RNAs, using cruder preparations of TAB (15). The maximum number of complexes formed is 3, irrespective of the length and conformation of the RNA; furthermore, the lack of partially occupied intermediate states indicates strong cooperativity of TAB-TAB interactions on the RNA. The results are consistent with a binding stoichiometry (RNA-TAB) of 1:1, 1:2, or 1:3, depending on factor concentration. Assuming that TAB binds as a dimer, up to 6 molecules of the 15-kDa subunit are potentially capable of aggregating on a single binding site on the RNA.

An important question is whether the binding site of TAB on tRNA coincides with the import signal. The precise RNA structural features directing import are not yet known, but one determinant resides in the D stem-loop region (12). Accordingly, a "half-tRNA" molecule containing nucleotides 1–36 of $tRNA^{Tyr}$ (including the D stem-loop and flanking anticodon and acceptor stem sequences) was tested for TAB binding *in vitro*. As shown in Fig. 4*B*, specific, mutifactor binding was observed; the pattern is consistent with an initial interaction of lower affinity with the D stem-loop (hence the presence of unreacted RNA), followed by cooperative binding of more TAB molecules to the preformed complex.

Immunochemical Localization of TAB—Polyclonal antibody against the 15-kDa subunit reacted with a single species on Western blots of S-100 extracts (Fig. 5); no signal was obtained with normal serum or IgG.[2] Immunofluorescent staining of promastigotes with this antibody revealed a cytoplasmic location of TAB (Fig. 5*A*), with the following notable features: 1) exclusion of nucleus and flagellum (exclusion is not evident in the ethidium bromide-stained nuclei but was observed in the absence of the DNA stain); 2) concentration of FITC fluores-

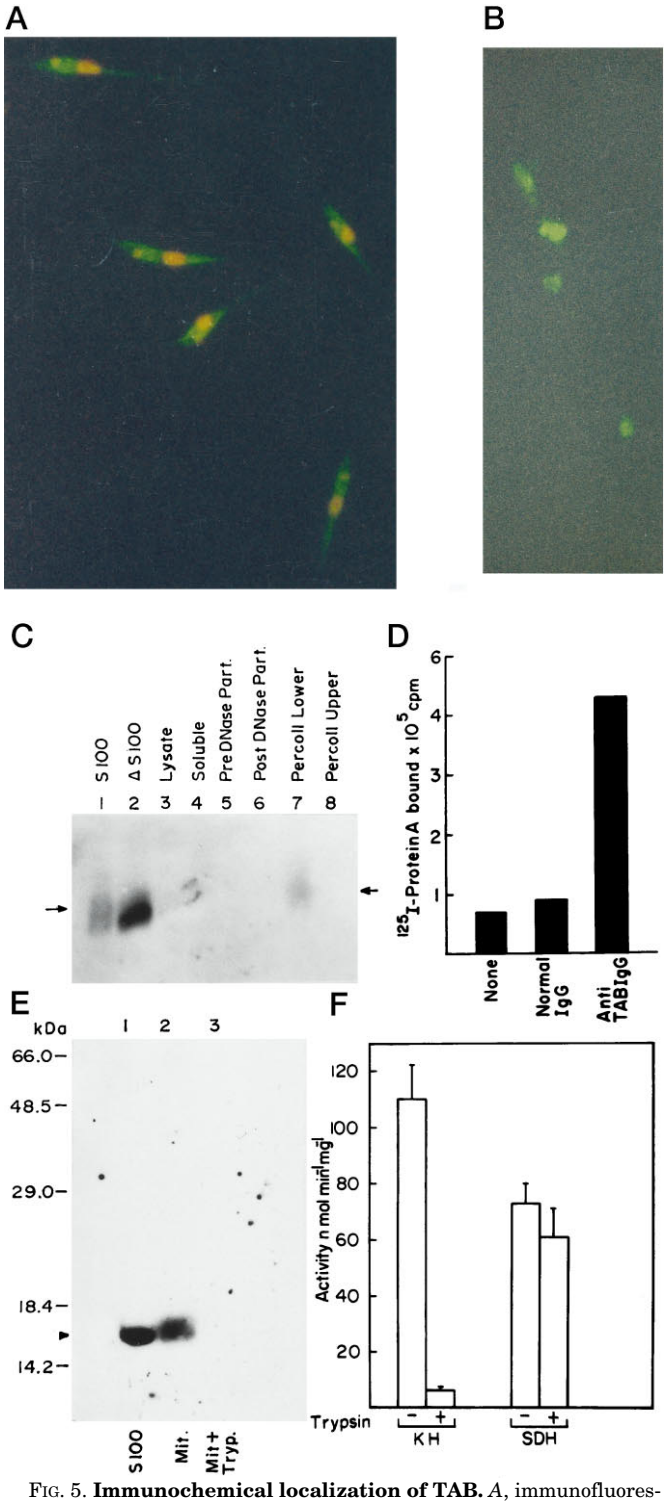

FIG. 5. **Immunochemical localization of TAB.** *A*, immunofluorescence of fixed promastigotes doubly stained with anti-TAB serum plus FITC-conjugated second antibody (*green*), and ethidium bromide (*orange*) photographed using FITC filter at × 1000 magnification. The nuclei and anteriorally lying kinetoplasts are distinguishable. *B*, immunofluorescence of native mitochondria stained with anti-TAB IgG plus FITC-conjugated second antibody (magnification, × 1000). *C*, Western blot analysis of subcellular fractions. *Lanes 1* and *2*, Triton-soluble S-100 or heat-treated S-100 (10 μg each), respectively. *Lanes 3–8*, 50 μg each of the following: hypotonic lysate; soluble fraction (concentrated by ammonium sulfate precipitation (0–80% cut) followed by dialysis against DB; pre-DNase particulate fraction; post-DNase particulate fraction; lower Percoll band of mitochondria; upper Percoll band (plasma membranes and cell debris) concentrated by ultracentrifugation at 83,000 × g_{av} for 30 min. Proteins were transferred to nitrocellulose and probed with anti-TAB antiserum plus ^{125}I-labeled protein A. Positions of the 15-kDa band (*arrowhead*) were determined with markers run on either side; the apparently high position of the band in *lane 7* is due to a gel artifact. *D*, immunodecoration of native mitochondria. Bovine serum albumin-blocked mitochondria (80 μg of protein) were incubated without IgG (*left*), with normal IgG (*center*) or anti-TAB IgG (*right*), followed by incubation with ^{125}I-protein A. *E*, Western blot analysis of proteins in S-100 extract (20 μg, *lane 1*), intact mitochondria (*Mit.*) (50 μg, *lane 2*) and trypsinized mitochondria (50 μg, *lane 3*), transferred to a polyvinylidene difluoride membrane and probed with anti-TAB IgG (10 μg/ml) and ^{125}I-labeled protein A. *F*, assay of kynurenine hydroxylase (*KH*) and succinate dehydrogenase (*SDH*) in native or trypsinized mitochondria. Activities are shown as nanomole of substrate converted per min/mg mitochondrial protein. *Bars* show the mean of four independent experiments; standard deviations are shown as *vertical lines*.

cence in many cells in the region anterior to the nucleus, coinciding with the position of the single kinetoplast-mitochondrion, marked by the ethidium bromide fluorescence of the compact kinetoplast DNA discs. This typically anterior position of TAB suggested its association with mitochondria, but other cytoplasmic locations could not be excluded.

Therefore, the content of TAB in equal amounts of various subcellular fractions obtained during isolation of mitochondria was monitored by the Western blot assay. Briefly, the method consists of hypotonic lysis of cells in the absence of detergents, separation of the particulate and soluble fractions, digestion of nuclear DNA, and Percoll gradient centrifugation (13, 17). As shown in Fig. 5*C*, the lower Percoll band of mitochondria was clearly enriched for TAB, as expected of its mitochondrial location. Heavy exposure of this gel showed faint bands in the various particulate fractions, but the signals obtained were always low compared with those obtained with Triton-solubilized S-100 extracts (Fig. 5, *C* and *E*), possibly due to the presence of interfering cellular components or an inadequacy of the fractionation procedure.

Native mitochondria complexed with anti-TAB but not normal IgG, could be immunodecorated with ^{125}I-labeled protein A (Fig. 5*D*) or with FITC-conjugated second antibody (Fig. 5*B*). Limited trypsinization of mitochondria resulted in release and/or degradation of TAB (Fig. 5*E*). Under these conditions of trypsin treatment, the outer membrane marker kynurenine hydroxylase was completely sensitive, but the inner membrane marker succinate dehydrogenase was relatively resistant (Fig. 5*F*). The accessibility of TAB in native mitochondria to antibody and protease indicates its association with the outer mitochondrial membrane.

Inhibition of Import by Anti-TAB Antibody—Mitochondria were preincubated with normal or immune IgG, washed free of excess IgG, and assayed for RNA import. Anti-TAB IgG inhibited import of $tRNA^{Tyr}$ transcript by more than 98% but normal IgG had no significant effect (Fig. 6*A, lanes 1–3*). Identical results were obtained for import of antisense transcripts (Fig. 6*B, lanes 1–3*). We further examined whether this inhibitory effect was at the level of receptor binding or transfer of RNA through the import channels. Anti-TAB IgG, but not normal IgG, inhibited binding of both $tRNA^{Tyr}$ transcript (Fig. 6*A, lanes 4–6*) and antisense RNA (Fig. 6*B, lanes 4–6*) to the mitochondrial surface by more than 90%. The specific abrogation of import as well as mitochondrial surface binding of RNA by monospecific antibody indicates a role of TAB as the major, if not the sole, import receptor or carrier.

DISCUSSION

The *in vitro* system described in this and previous reports (13, 14), is relatively inefficient, with only about 10% of the bound RNA being imported. This could be due to a number of reasons, *e.g.* the method of isolation of mitochondria, which includes exposure to hypotonic conditions and shear forces; rapid hydrolysis of ATP by mitochondrial ATPase; structure

and/or sequence features of the RNA; absence of stimulatory factors; and inadequacies of the assay method such as rupture of the vesicles during post-import washes or insufficient inactivation of RNase. Nevertheless, the present system is capable of importing isolated homologous tRNA in an ATP-dependent and saturable manner (Fig. 1) and shows discrimination between $tRNA^{Tyr}$ and $tRNA^{Gln}$(CUG), as observed *in vivo* (8). These characteristics make it suitable for detailed studies of the import mechanism.

Immunochemical experiments presented here (Figs. 5 and 6) identify, for the first time, a role of the 15-kDa TAB protein as a receptor or carrier for mitochondrial RNA import. TAB is associated with the outer mitochondrial membrane, as judged by its accessibility to RNA, trypsin, and antibody (Figs. 2 and 5). However, it remains to be seen whether TAB is anchored to the membrane directly, via a hydrophobic stretch of amino acid residues, or indirectly, through interaction with some other outer membrane protein. The absence of an N-terminal methionine indicates that TAB is derived by proteolytic processing of a larger precursor. Removal of a transit sequence is one possibility. A BLASTP search of the Swiss Prot data base revealed 70% identity of the N-terminal decapeptide of TAB with the mitochondrial membrane protein dihydroorotate dehydrogenase of *Plasmodium falciparum* (23); the significance of this homology is unclear at present.

The structure of the recognition site of TAB on the RNA, and the mode of binding, are of relevance to the import function of the protein: if TAB is an import receptor, the binding site and import signal should coincide. We have previously shown a preference of TAB for oligopurine motifs on antisense RNA (15). Similar motifs are present in the D stem-loops of importable tRNAs, leading to the hypothesis that this region constitutes part or the whole of the import signal (13, 14). This was recently demonstrated *in vivo* by D stem-loop exchange between an imported and a non-imported tRNA (12). The observation that TAB binds to half-tRNA (Fig. 4) is therefore in keeping with its role as receptor or carrier.

In solution, and under factor excess conditions, up to 3 molecules of TAB bind cooperatively to each RNA molecule (Fig. 4). It is difficult to correlate this mode of binding to that which occurs on the mitochondrial surface. Nevertheless, it is interesting to note that Scatchard analysis of RNA binding to membrane-bound TAB revealed multiple binding affinities (14), possibly reflecting cooperativity. Under RNA excess conditions, the bound RNA concentration is about 4 nM, while the total receptor concentration (including low and high affinity sites) is 5.1 nM (14). This corresponds to an RNA:receptor ratio of 0.8:1. It is possible that oligomerization of RNA-bound TAB on the mitochondrial surface occurs prior to import, a situation analogous to the dimerization of certain cell-surface receptors induced by bivalent ligands or antibody.

At least one other mitochondrial surface-bound tRNA-binding protein of 22 kDa was detected on Northwestern blots (Fig. 2). Antisense RNA does not detectably bind to this protein under the same conditions (14), suggesting that the RNA motifs recognized by the 15- and 22-kDa proteins are different. The role, if any, of this protein in RNA import remains obscure. Nevertheless, it is interesting to note that, whereas the 22-kDa band appears to be the major tRNA-binding species on blots (Fig. 2), anti-TAB antibody inhibits binding of tRNA to the surface of intact mitochondria by more than 90% (Fig. 6), although there is no cross-reaction between the antibody and the 22-kDa species (Fig. 5). One explanation of this discrepancy is that transfer of tRNA from TAB to the 22-kDa protein occurs prior to import. Further studies of the 22-kDa factor are warranted by these observations.

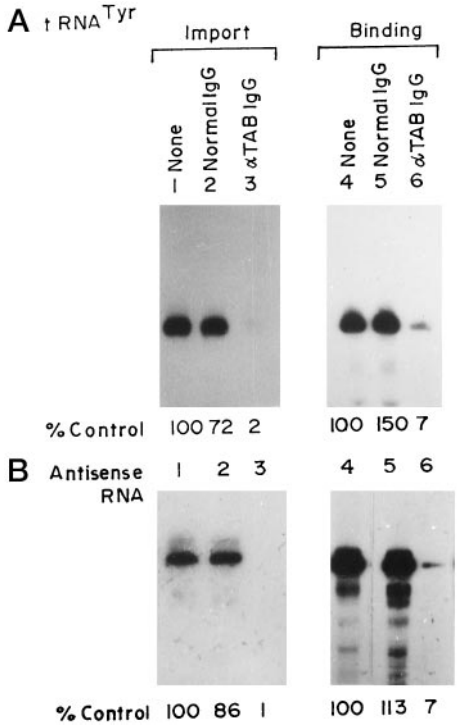

FIG. 6. **Inhibition of RNA import by anti-TAB IgG.** Import (*lanes 1–3*) and binding (*lanes 4–6*) assays using ^{32}P-labeled $tRNA^{Tyr}$ transcript (*panel A*) or antisense RNA (*panel B*) were performed with untreated mitochondria (*lanes 1* and *4*), or mitochondria preincubated with normal IgG (*lanes 2* and *5*) or anti-TAB IgG (*lanes 3* and *6*). Quantitation of bands was done by liquid scintillation counting in *A* and by densitometric scanning in *B*.

Two models have been proposed to explain the import of a highly negatively charged RNA molecule through the double mitochondrial membrane. The co-import model (24) supposes RNA, complexed with a soluble carrier protein, to pass through protein import channels utilizing an import signal on the carrier. Alternatively, RNA would be directly transferred through recognition by specific membrane-bound receptors. In yeast, mature $tRNA_1^{Lys}$ apparently has to be aminoacylated before it can be imported (25). By contrast, mutant $tRNA^{Tyr}$ precursors which cannot be aminoacylated have been observed in *Trypanosoma brucei* mitochondria (26). The import of unspliced $tRNA^{Tyr}$ *in vitro* (Fig. 1) confirms that splicing is not a prerequisite for import. tRNA import in yeast requires a cytosolic carrier (3), presumably the cognate aminoacyl tRNA synthetase, whereas the *Leishmania* system has no such requirement, and cytosolic fractions have no effect.[2] Instead, one or more membrane-bound RNA-binding proteins mediate import. The involvement of the protein import receptor MOM19 in yeast tRNA import was implied by mutant analysis (27), lending support to the co-import model. In view of the known requirement of unfolding of the polypeptide chain during translocation (Ref. 2 and references therein), this model, invoking non-covalent RNA-protein complexes, has serious mechanistic difficulties. Our experiments (14) on the salt and polyanion sensitivities of the RNA-mitochondrion complexes argue against a similar mechanism in *Leishmania.* All in all, it appears that *Leishmania* mitochondria use a specialized high-affinity membrane-bound receptor system for direct import of large numbers of tRNAs to maintain mitochondrial viability.

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