Chapter VIII

RIC8B is another member of the RIC family

BN PAGE of RIC8A knockdown extract when subjected to western blotting using anti RIC8B, C and D antibody revealed the presence of RIC8B only in the smaller complex (R\textsuperscript{8A}) (Fig. 90).

![Fig. 90](image)

SDS PAGE analysis of R\textsuperscript{8A} showed a coomassie stained band at the position of 21 kD which when western blotted with anti-RIC8B antibody confirmed the presence of RIC8B in the 21 kD band of RIC (Fig. 90).

![Fig. 91](image)

The result showed that RIC8B is another protein of RIC family and knockdown of RIC8A does not inhibit the assembly of RIC8B into RIC.
Results

Cloning and expression of RIC8B

RIC8B was cloned in the same manner as RIC8A. Sense and antisense primers corresponding to the open reading frame of RIC8B were used to amplify the intact full length gene from *L. tropica* strain UR6 genomic DNA. The sense primer was designed to have a BamHI restriction site at the 5' end in frame with the coding sequence of the insert; the antisense primer had an inframe stop codon followed by a Sall site. The amplified fragment was cloned into TA vector pTZ57R. Subsequently RIC8B was expressed in *E. coli* BL21 host cell following transfer of the gene fragment from pTZ57R to pGEX4T1 vector. Polyclonal antibodies were raised against the purified recombinant RIC8B protein in BALB/c mice.

Comparative analysis of RIC8B

Full length RIC8B insert cloned into pTZ57R (MBI Fermentas) was sequenced from both ends using automated method employing dye terminator technology in an Applied Biosystems, Version 3.0 apparatus.

Upon comparison, the *L. tropica* UR6 sequence was found to be identical to that of the *L. major* friedlin sequence. Thus there was no change in the protein sequence. Blast analysis did not show any sequence similarities with any known protein.

Genomic organization of RIC8B

*L. major* friedlin genome sequence shows that this 603 bp single copy gene (systematic location LmjF04.0630) has Ncol, Sall, EcoRI and Kpnl restriction sites and is localized on chromosome 4 (Fig. 92).

![Fig. 92: Context Map: Genomic organization of RIC8B](image-url)
Preparation of recombinant proteins and antibodies

As described in RIC8A, RIC8B was similarly first cloned in pTZ57R TA vector, transferred to pGEX4T1 and then expressed in *E. coli* BL21 cells. Polyclonal antibody was raised in BALB/c mice.

Conditional knockdown of RIC8B

RIC8B was cloned in antisense orientation in pGET vector in the same way as RIC8A and the recombinant clone (Fig. 93) was electroporated into Leishmania 13-90 host strain.

![Figure 93: Schematic diagram of the expression cassette of knockdown vector pGET - (AS) RIC8B.](image)

The transformants were selected on semisolid agar containing G418, Hygromycin, and 2'5 μg/ml Bleomycin. Clones were grown in M199 medium containing the same antibiotic.

Effect of tetracycline on the growth of pGET (RIC8B AS) transformants

Cultures were induced with 1 μg/ml Tetracycline and cell growth was monitored. (Fig. 94). In the absence of tetracycline, the antisense-RIC8B transformants grew normally with a doubling time of ~24 h at 22 °C, which is close to the doubling time for cells transfected with the empty vector. Whereas growth of the antisense transformants ceased after 72 hours; promastigotes became rounded and non-viable.
Results

Growth of control cells transformed with the empty vector was not affected by tetracycline. Withdrawal of tetracycline after 96 hours resulted in resumption of growth after a lag of 3–4 days, suggesting that the knockdown is reversible or that a few cells escape knockdown. Thus, tetracycline-induction of expression of the insert has a lethal effect on cell growth (Fig. 94).

![Graph showing effect on cell growth at 22°C. Closed squares, pGET-transformed L. tropica, +tet; open squares, pGET(AS)RIC8B transformed, + tet.]

Knockdown of RIC8B mRNA and protein

Similar to RIC8A, the status of the RIC8B mRNA in the knockdown strains was determined in the following way: uninduced or induced cells (72 hours after induction) were harvested, lysed, and separated into soluble (cytosolic) and particulate (mitochondrial) fractions. RNA was isolated from the crude mitochondria after treatment with DNase and RNase. Reverse transcription of RNA from $10^5$ cells was performed with Superscript II reverse transcriptase and the appropriate primer, as follows: (i) for antisense RNA, the sense primer -(O-89), corresponding to the 5' end of the coding region of the RIC8B gene; (ii) for the mRNA, the antisense primer -(O-90), complementary to the 3' end of the gene.

It was observed that in the presence of tetracycline, RIC8B antisense RNA was induced, with the concomitant down regulation of the corresponding mRNA (Fig. 95).
Results

To determine the status of RIC8B protein, total cellular and mitochondrial fractions (100µg/lane) of both induced and un-induced cells were resolved by 12% SDS-PAGE. The separated proteins were then transferred to a nitrocellulose filter and probed with α-RIC8B antibody. As a control, the levels of F1β and Complex II iron-sulfur (Fe-S) protein were also determined by probing parallel blots with respective antibodies. It was seen that although there was no change in the total protein profile, RIC8B protein became nearly undetectable in the induced fractions, both cellular and mitochondrial. The levels of the other mitochondrial proteins such as F1β and Fe-S protein were unaffected (Fig. 96).

<table>
<thead>
<tr>
<th>Total</th>
<th>Mitochondrial</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIC8B</td>
<td>RIC8B</td>
</tr>
<tr>
<td>F1β</td>
<td>F1β</td>
</tr>
<tr>
<td>Fe-S</td>
<td></td>
</tr>
<tr>
<td>Tet</td>
<td>- +</td>
</tr>
</tbody>
</table>

Fig. 96: Knockdown of RIC8B in induced cells (+tet). Immunoblots were done to detect the level of RIC8B in left: total cellular fraction and right: mitochondrial fraction of induced and uninduced cells.

Mitochondrial integrity in the induced cells was checked by treating the mitochondrial fraction was with trypsin (20µg/ml, 20 mins, 4°C) either in the presence or in the
absence of 0.5% Triton-X-100. The resulting extract was electrophoresed, transferred and then blotted with anti-F1β antibodies. It was seen that inner membrane proteins like the F1β protein remained inaccessible to trypsin unless released by Triton-X-100 (Fig. 97). Thus, RIC8B knockdown had no effect on either the integrity of the mitochondria or the presence of other inner mitochondrial membrane proteins.

**Fig. 97: Immunoblots of TX-100 and Trypsin treated mitochondrial fraction of induced and uninduced cells**

| Tx-100 | + |
| Trypsin | + |
| F1β | - |
| Tet | - |
| Mitochondrial | |

**Effect of RIC8B knockdown on tRNA import in Leishmania**

To determine the effect of RIC8B knockdown on the level of endogenous mitochondrial tRNA imported from the cytosol, total RNA was isolated from the mitochondrial fraction of ~10^2 induced and uninduced cells. The RNA was reverse transcribed using the primers complementary to the 3’ end of the 6 tRNA genes (tRNA_Tyr, tRNA_Trp, tRNA_Arg, tRNA_Ile, tRNA_Val, tRNA_Met-e). RIC8B knockdown had a severe effect on mitochondrial tRNA import. The levels of all 6 nucleus-encoded tRNAs viz. tRNA_Tyr, tRNA_Trp, tRNA_Arg, tRNA_Ile, tRNA_Val, tRNA_Met-e (both type I & type II tRNAs) in the mitochondria were reduced to <5% with respect to that of the uninduced cells (Fig. 98 & 99).

Thus, *in vivo* knockdown of RIC8B results in depletion of both the mitochondrial type I and type II tRNAs.
Results

Fig. 98: Level of mitochondrial type I tRNAs in RIC8B knockdown cells. RT-PCR assays were done to detect the level of endogenous tRNAs in mitochondrial fractions.

However there was no corresponding significant change in the level of the cytosolic tRNAs (Fig. 100).

Fig. 99: Level of mitochondrial type II tRNAs in RIC8B knockdown cells. RT-PCR assays were done to detect the level of endogenous tRNAs in mitochondrial fractions.

Fig. 100: Level of cytosolic type I (tRNA\textsubscript{fri}) and type II (tRNA\textsubscript{fri}) tRNAs in RIC8B knockdown cells.
Results

Effect of RIC8B knockdown on mitochondrial translation

RIC8B deficiency leads to an imbalance in the tRNA pool in the mitochondria thereby severely affecting different mitochondrial processes in vivo. This in turn causes various downstream defects in mitochondrial function.

The effect of RIC8B knockdown on mitochondrial translation was tested by incubating $10^8$ cells in 100µl of methionine-deficient RPMI medium 1640 (Invitrogen) and 40µCi of $[^{35}S]$methionine, in the presence of cycloheximide and, in the presence or absence of chloramphenicol. The resulting mixture was incubated at 22°C for 4 hours with shaking. After 4 hours, mitochondrial fractions were recovered from the labeled cells and treated with DNase and RNase. The proteins were solubilized in SDS-PAGE sample buffer for 1 hour at 37°C and then electrophoresed in a 12% SDS-PAGE. It was observed that although cytosolic protein synthesis was normal in the induced cells (+Tet), cycloheximide-resistant, chloramphenicol-sensitive mitochondrial translation was almost completely abolished (Fig. 101).

![Fig. 101: $[^{35}S]$ Methionine labeled proteins from uninduced or tet-induced pGET-(AS) RIC8B transformed Leishmania 13-90 cells cultured in presence of cycloheximide. (Tet: tetracycline; CAM: chloramphenicol; CHX: cycloheximide).](image)

Effect of RIC8B knockdown on respiratory functions

The oxygen consumption of $10^9$ cells in 3 ml of PBS-glucose was measured at 30°C in a thermostatically controlled chamber (Biological Oxygen Monitor YSI 5300A) equipped with a Clarke type oxygen electrode. The rate of $O_2$ consumption of RIC8B
knockdown cells was reduced to ~20% of that of normal. (Wild type cells = 1.2 fmoles/min/cell; RIC8B knockdown cells = 0.22 fmoles/min/cell).
The cytochrome c oxidase activity was also severely reduced in the RIC8B knockdown cells. The number of cells positive for cytochrome oxidase (or Complex IV) was reduced to ~10% of the uninduced value.

**Effect of RIC8B knockdown on respiratory complexes**

When the respiratory complexes from knockdown cells were analyzed by BN PAGE, one small complex, instead of the usual four, was observed (Fig. 102A). This complex when resolved in an SDS-PAGE revealed the subunit profile of a subcomplex of RIC (R<sup>8B</sup>; Fig. 102B).

Western blotting using anti-RIC8B antibody showed that this subcomplex lacked RIC8B (Fig. 102C); the residual Coomassie-stained 21-kDa band is that of subunit RIC8A was proved by western blotting with anti-RIC8A antibody (αRIC8A) (Fig. 102D). Moreover RIC1, RIC2, RIC5 and RIC7 were missing in the subcomplex which indicated that these failed to be assembled in the absence of RIC8B. A lack of major mitochondrially encoded subunits also accounts for the failure to assemble Complexes V, III and IV.

![Fig. 102: (A) BN PAGE profile of mitochondrial extracts of wild type (-Tet) and RIC8B knockdown (+Tet) cells. (B) Coomassie stained SDS PAGE profile of RIC band and R<sup>8B</sup>. (C) Western blot using anti-RIC8B antibody. (D) Western blot using anti-RIC8A antibody.](image-url)
Results

RIC8B interacts with RIC1

Purified recombinant RIC1, RIC8B and proteoliposomes were reconstituted and the reconstituted products were run on a SDS-PAGE followed by western blotting with anti-RIC1 and RIC8B. The result showed that RIC1 alone was incapable of incorporation into proteoliposome whereas in presence of RIC8B it got incorporated (Fig. 103). The result also showed that RIC8B alone was capable of incorporation into liposome.

![Figure 103: Purified RIC8B, RIC1 and liposomes were incubated. The products were run on BN PAGE and subsequently western blotted with anti-RIC1 antibody (left) and anti-RIC8B antibody (right).](image)

To further prove this interaction ATPase activity of RIC1 was used. Reconstituted RIC1, RIC8B and proteoliposomes when assayed for ATPase activity, RIC1 only in presence of RIC8B showed ATPase activity (Fig. 104); this was because liposome-bound RIC8B interacted with RIC1 and RIC1 in the subsequent RIC8B-RIC1 complex showed ATPase activity after washing.

![Figure 104: Autoradiography of the TLC showing ATPase activity of the RIC1-RIC8B complex in liposome.](image)
Discussion

Knockdown of RIC8A revealed the presence of another 21 kD component in the RIC. This was subsequently found to be RIC8B which is a 603 bp single copy gene. Knockdown of this protein resulted in the complete loss of the tRNA import process in *Leishmania* mitochondria which in turn affected the mitochondrial translation. The effect of RIC8B knockdown on RIC resulted in a drastic change in the subunit profile. Subunits RIC1, RIC2, RIC4B, RIC5, RIC7 and RIC9 were absent from the knockdown complex. This could be due to two effects viz. shut down of the mitochondrial translation and interaction of RIC8B with other subunits. RIC8B was also found to incorporate itself into liposome and interacts with RIC1. Thus RIC8B is a very important assembly component of RIC.