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The 2–5A/RNase L/RNase L Inhibitor (RNI) Pathway Regulates Mitochondrial mRNAs Stability in Interferon α-treated H9 Cells

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Interferon α (IFNα) belongs to a cytokine family that exhibits antiviral properties, immuno-modulating effects, and antiproliferative activity on normal and neoplastic cells in vitro and in vivo. IFNα exerts antitumor action by inducing direct cytotoxicity against tumor cells. This toxicity is at least partly due to induction of apoptosis. Although the molecular basis of the inhibition of cell growth by IFNα is only partially understood, there is a direct correlation between the sensitivity of cells to the antiproliferative action of IFNα and the down-regulation of their mitochondrial mRNAs. Here, we studied the role of the 2–5A/RNase L system and its inhibitor RLI in this regulation of the mitochondrial mRNAs by IFNα. We found that a fraction of cellular RNase L and RLI is localized in the mitochondria. Thus, we down-regulated RNase L activity in human H9 cells by stably transfecting (i) RNase L antisense cDNA or (ii) RLI sense cDNA constructions. In contrast to control cells, no post-transcriptional down-regulation of mitochondrial mRNAs and no cell growth inhibition were observed after IFNα treatment in these transfectants. These results demonstrate that IFNα exerts its antiproliferative effect on H9 cells at least in part via the degradation of mitochondrial mRNAs by RNase L.

IFNαs are negative regulators of cell proliferation through induction of cell cycle arrest and apoptosis, which has also been suggested to be of central importance in IFNα antitumor action. IFNα (type I family) has emerged as a tumor suppressor protein. A number of clinical trials were thus carried out in cancer therapy with IFNα alone or in combination with other negative regulators of cell proliferation (4, 5). It has been shown to be effective against hematological malignancies including hairy cell leukemia, chronic myelogenous leukemia, and cutaneous T-cell lymphoma (6). IFNα has been used successfully in cancer therapy, but development of resistance to IFNα therapy has often been observed in patients. Although the molecular basis of the inhibition of cell growth by IFNα is partially understood, there is a correlation between the sensitivity of cells to the antiproliferative action of IFNα and the down-regulation of their mitochondrial mRNAs and the impairment of mitochondrial function upon IFNα treatment (7, 8).

In this work, we have studied the role of one of the enzymatic pathways induced by IFNα, the 2–5A/RNase L pathway, in the regulation of mitochondrial mRNAs. The 2–5A/RNase L pathway is a RNA degradation pathway (9). IFNα induces the expression of 2–5A synthetase(s) which, upon activation by double-stranded RNA, convert ATP into an unusual series of oligomers known as 2–5A (10, 11). 2–5A activates RNase L, a latent endoribonuclease, which inhibits protein synthesis by cleaving mRNAs of Upn-p (uracil cytidine, the nucleotide sequence of mRNA recognized by RNase L) sequences (12, 13). RNase L activity is also regulated by RNase L inhibitor (RLI), which inhibits 2–5A binding to RNase L, thereby switching off the 2–5A/RNase L system (14).

We postulated that RNase L may participate in the antiproliferative effect of IFNα by playing an important role in the IFNα-induced down-regulation of mitochondrial mRNAs, which leads to a decrease in cellular ATP levels and suppression of mitochondrial function (7, 8). In this report, we demonstrate the presence of RNase L and RLI in mitochondria. Furthermore we show that in IFNα-treated H9 cells, the level of mitochondrial mRNAs is a function of RNase L activity in the mitochondria. Inhibition of RNase L can prolong the stability of mitochondrial mRNAs.

EXPERIMENTAL PROCEDURES

Cells, IFN, and Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP)—Human H9 cells (lymphoma, cutaneous T lymphocyte; American Type Culture Collection (ATCC): HTB-176), human HeLa cells (adenocarcinoma, cervix; ATCC: CCL-2), and human Daudi cells (Burkitt’s lymphoma, B lymphoblast; ATCC: CCL-213) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum. Cells were incubated at 37 °C in a 5% CO2, 95% air atmosphere. Cell growth was determined by counting viable cells as determined by trypan blue exclusion. Human IFNα (IFNα2) was used at 500 units/ml. The CCCP was added to culture media at 20 μM for the same period of time as IFNα treatment. The experiments were done in triplicate, and the S.D. is indicated on the plots.

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¶ The abbreviations used are: IFN, interferon; RLI, RNase L inhibitor; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TIM, translocase complex in the inner membrane; TOM, translocase complex in the outer membrane.
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Cell Extracts and Isolation of H9 Mitochondria—H9 cells were washed twice in ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 2 mM KCl, 8 mM Na$_2$HPO$_4$, pH 7.4), pelleted, resuspended in 2 volumes of hypotonic buffer (0.5% (v/v) Nonidet P-40 (NP-40), 20 mM Heps pH 7.5, 10 mM potassium acetate, 15 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotdin, 150 μg/ml leupeptin), incubated 10 min on ice, disrupted with a Dounce homogenizer, and centrifuged at 10,000 × g at 4°C. The obtained supernatant was designated post-nuclear extract. For mitochondria isolation, H9 cells (2.0 × 10$^6$ cells) were washed twice in ice-cold PBS, pelleted, and resuspended in 5 ml of a 10 mM Tris-HCl, pH 6.7, 10 mM KCl, 0.15 mM MgCl$_2$ solution. After incubation for 10 min on ice, cells were disrupted with a Dounce homogenizer and, after the addition of sucrose (0.25 M final concentration), centrifuged at 10,000 × g for 5 min to remove the nuclei. The supernatant was complemented with K-EDTA (1 mM final concentration) and centrifuged at 10,000 × g for 10 min at 4°C. The mitochondrial pellet was resuspended in cold buffer A (0.25 M sucrose, 10 mM Tris acetate, pH 7.0). The supernatant was named the cytoplasmic extract (without mitochondria). The mitochondria were purified on a 30% (v/v) Percoll (Amersham Pharmacia Biotech) cushion in buffer A supplemented with 1 mM K-EDTA. The mitochondria were collected from the cushion and centrifuged to eliminate Percoll. Mitochondria were lysed by sonication, and the total mitochondrial extract was used for further experiments. The protein concentration was determined by Bradford reaction (15).

Expression Vectors and Transfections—The coding sequences of human RLI cDNA (14) and human RNase L cDNA (13) were subcloned in pcDNA3neo (Invitrogen) by standard procedures (16). RLI sense/pcDNA3neo (7 μg), RNase L antisense/pcDNA3neo (7 μg), and the empty vector (7 μg) were transfected in H9 cells by electroporation as previously described (17). Transfected H9 cell clones were isolated by dilution of 1 cell/slot in 96-slot plates in the presence of 1 mg/ml Geneticin (Life Technologies, Inc.). Two clones were selected for each construction and named RLI S1 and RLI S2 for clones expressing sense RLI cDNA and RNase L AS1 and RNase L AS2 for clones expressing RNase L cDNA in the antisense orientation. An empty pcDNA3 vector-transfected clone was used as a control.

Inactivation of Mitochondrial DNA and Mitochondrial cDNA Synthesis—Mitochondrial DNA was isolated from human Daudi cells by lysis in a 2% (w/v) SDS solution, two phenol dichloromethane extractions, and ethanol precipitation. Polymerase chain reaction was performed directly on mitochondrial DNA to amplify cytochrome b, ATPase 6, and cytochrome oxidase subunit II cDNA. Primer 1 (5′-GGGAATTCACAGGGACACCAATGACGCCCA-3′) and primer 2 (5′-GGGAATTCACAGGGACACCAATGACGCCCA-3′) were used to amplify cytochrome b cDNA. Primer 3 (5′-GGGAATTCACAGGGACACCAATGACGCCCA-3′) and primer 4 (5′-GGGAATTCACAGGGACACCAATGACGCCCA-3′) were used to amplify ATPase 6 cDNA. Primer 5 (5′-GGGAATTCACAGGGACACCAATGACGCCCA-3′) and primer 6 (5′-GGGAATTCACAGGGACACCAATGACGCCCA-3′) were used to amplify cytochrome oxidase subunit II cDNA.

RNA Analysis—RLIS1, RLIS2, RNase L AS1, RNase L AS2, and control cells were plated at 2.0 × 10$^5$ cells/ml, treated for 24 or 48 h by IFNα (500 units/ml), and collected for RNA extraction using the guanidine thiocyanate-lithium chloride procedure (18). Total RNA (20 μg) was analyzed by Northern blot (16). After transfer onto nylon membranes (Amersham), rRNA were revealed with 0.5 μl (sodium acetate) pH 5, 0.04% (w/v) methylene blue. The nylon membranes were then incu-
bated with the appropriate [32P]cDNA probes (as indicated in the figure legends) synthesized using the Multiprime radiolabeling kit (Life Technologies, Inc.). After autoradiography on a PhosphorImager 445-SI (Molecular Dynamics), the mRNA were quantified by image analysis with the Image Quant program (Molecular Dynamics). The experiments were done in triplicate, and the S.D. is indicated on the plots.

Western Blot—Proteins (100 µg of post-nuclear extract, 100 µg of cytoplasmic extract, 100 or 200 µg of mitochondrial extract) were fractionated by SDS/polyacrylamide gel electrophoresis and transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell) according to Towbin et al. (19). For detection with antibody, the nitrocellulose membranes were incubated in PBS supplemented with 10% (w/v) skim milk powder for 30 min and then soaked overnight at 4 °C in the same buffer with the appropriate antibodies: rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) at 1:3000 dilution, rabbit polyclonal anti-RIPL at 1:5000 dilution (20), rabbit polyclonal anti-RNase L at 1:1000 dilution (20), rabbit polyclonal anti-MITL at 1:5000 dilution. The membranes were washed with PBS supplemented with 0.05% (v/v) Tween 20 and incubated for 1 h at room temperature with donkey anti-rabbit IgG antibody or with sheep antimouse IgG antibody conjugated to horseradish peroxidase at 1:2000 dilution (Amersham Pharmacia Biotech). Chemiluminescence was generated by reaction with the PerkinElmer Life Sciences enhanced chemiluminescence kit. The autoradiographies were scanned, and proteins bands were quantified by image analysis with the Intelligent Quantifier program (BioImage Systems Corp.). The experiments were done in triplicate, and the S.D. is indicated on the plots.

Radioisotopic labeling of RNase L—The radiolabeled 2-5A,3'-[32P]PpCp probe was oxidized at its 3' end (21). This modified probe can be covalently linked to amine groups on RNA L and allows analysis by denaturing gel electrophoresis as initially described by Wreschner et al. (22) with our modifications (23). Post-nuclear extracts (100 µg), cytoplasmic extracts (100 µg), or mitochondrial extracts (100 or 200 µg) were incubated with the oxidized 2-5A,3'-[32P]PpCp probe for 30 min in ice and for a further 20 min at room temperature with sodium cyanoborohydride (20 mM final concentration). Proteins were analyzed by 10% (v/v) SDS/polyacrylamide gel electrophoresis (24). Labelled proteins were visualized after autoradiography, and proteins bands were quantified by image analysis with the Intelligent Quantifier program (BioImage Systems Corp.). The experiments were done in triplicate, and the S.D. is indicated on the plots.

Immunofluorescence—Human HeLa cells were cultivated at a concentration of 105 cells/ml in RPMI 1640 supplemented with 10% (v/v) fetal calf serum for 24 h. The cell culture medium was then changed to RPMI 1640 supplemented with 10% (v/v) fetal calf serum and 100 mM MitoTracker dye (red CM-H2XRos, Molecule Probes), incubated at 37 °C for 30 min, washed at 37 °C with PBS, and incubated for 5 min at room temperature in PBS, 3.7% (v/v) formalin. After several washes with PBS, cells were incubated in PBS, 0.1% (v/v) Triton X-100, 10% (v/v) bovine serum albumin for 20 min, then in PBS, 10% (v/v) bovine serum albumin for 1 h. Cells were further incubated for 1 h at room temperature with a rabbit anti-RNase L polyclonal antibody at 1:200 dilution, washed with PBS, incubated for 1 h at room temperature with fluorescein isothiocyanate isomer-conjugated goat anti-rabbit IgG at 1:50 dilution, washed with PBS, fixed with MOWIOL (2.4 g of MOWIOL 4–86 in 6 g of glycerol, 6 ml of water, 12 ml of 0.2 M Tris-HCl, pH 8.5), and analyzed by confocal microscopy on a Bio-Rad MRC 1024. The experiments were done in triplicate.

In Organello Pulse Chase—Immediately after purification, aliquots of the mitochondrial fraction (1 mg of mitochondrial protein) were resuspended in 0.5 ml of an incubation buffer containing 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K2HPO4, 0.05 mM EDTA, 5 mM MgCl2, 1 mM ADP, 10 mM glucose, 2.5 mM malate, 10 mM Tris/HCl pH 7.4, 1 mg/ml bovine serum albumin, and 20 µCi of [32P]UTP (800 Ci/mmol) (25). Incubation was carried out at 37 °C for 60 min, and the mitochondrial samples were pelleted at 13,000 × g for 1 min to remove non-incorporated [32P]UTP. The mitochondria were resuspended in the incubation buffer described above in the presence of a 200-fold excess of unlabeled UTP and incubated for increasing periods of time of as indicated in the figure legends. Mitochondrial RNAs were extracted twice with an equal volume of a phenol/dichloromethane/isooamy alcohol mixture (25:25:1, v/v) at room temperature. After ethanol precipitation of the aqueous phase and centrifugation, the nucleic acids pellet was dissolved in sterile water. The labeled transcripts from the different mitochondrial fractions were hybridized at 42 °C with mitochondrial cDNA spotted (5 µg) on a nitrocellulose membrane already prehybridized for 48 h in 50% (v/v) formamide, 50 mM NaH2PO4, pH 6.5, 0.8 mM NaCl, 1 mM EDTA, 2.5× Denhardt’s, 250 µg/ml salmon sperm DNA, and 500 µg/ml baker yeast tRNA. After 48 h of hybridization, the membranes were washed for 10 min at 20 °C with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% (w/v) SDS, 30 min at 65 °C with 0.2× SSC, 15 min at 37 °C with 2× SSC, RNase A (10 µg/ml), and 2 times for 30 min at 37 °C with 2× SSC (26). After autoradiography on a PhosphorImager 445-SI (Molecular Dynamics), the mRNAs were quantified by image analysis with the Image Quan program (Molecular Dynamics). The experiments were done in triplicate, and the S.D. is indicated on the plots.

In Organello Mitochondrial mRNA Stability in Presence of 2-5A—Transcription of mitochondrial RNA was done as described in the previous paragraph (25). The mitochondria were then resuspended in the incubation buffer described above in the presence of a 200-fold excess of unlabeled UTP and incubated for increasing periods of time as indicated in the figure legends in the presence or absence of 2-5A (100 µM). Mitochondrial RNA was extracted, spotted, and analyzed as described above.
down-regulation of mitochondrial mRNAs in IFN α mentioned in the introduction, our working hypothesis is that IFN α polyclonal antibody against RNase L. Twenty-four hours of treatment increased mitochondrial quantities of RNase L, but not RLI level (panels A and B). The absence of GAPDH in the mitochondrial extract (panels A and C) indicated that there was no cytoplasmic contamination during mitochondrial fractionation. As a positive control of mitochondria isolation and purity, we probed the Western blot for MRLP12, cytochrome b; ATP6, ATPase 6; COII, cytochrome oxidase subunit II. B, densitometric analysis of the gels shown in panel A. Data are presented as the log of RNA remaining versus time. A value of 1 corresponds to the log of the amount of RNA at time 0, before incubation with unlabeled UTP and 2-5A, or UTP alone. The S.D. obtained from three independent experiments are indicated by error bars.

RESULTS

RNase L and RLI Are Present in the Mitochondria—As mentioned in the introduction, our working hypothesis is that down-regulation of mitochondrial mRNAs in IFNα-treated cells could be due to RNase L activation. For RNase L and RLI to act as regulators of mitochondrial mRNA stability they must, first, be present in mitochondria and, second, RNase L must be able to degrade mitochondrial mRNAs. In keeping with this hypothesis we provide the first evidence for the presence of RNase L and RLI in isolated mitochondria (Fig. 1, panels A and B). RNase L was detected by radiocopy affinity labeling, because the level of RNase L present in the mitochondria is lower than the detection threshold level on Western blot, using our polyclonal antibody against RNase L. Twenty-four hours of IFNα treatment increased mitochondrial quantities of RNase L but did not affect RLI level (panels A and B). The absence of GAPDH in the mitochondrial extract (panels A and C) indicated that there was no cytoplasmic contamination during mitochondrial fractionation. As a positive control of mitochondria isolation purity, we probed the Western blot for MRLP12 protein, which is the mitochondrial mammalian homologue to chloroplastic and bacterial L12 ribosomal proteins.

To prove that RNase L and RLI are imported into mitochondria from the cytoplasm and that their detection in mitochondria is not due to nonspecific adsorption of these proteins on the outer mitochondrial membrane, CCCP was used to abolish the membrane potential required for polypeptide import through the inner mitochondrial membrane (28, 29). CCCP treatment of H9 cells decreased MRLP12 protein levels 4-fold, RNase L protein levels 8-fold, and RLI protein levels 2-fold in mitochondria, indicating that these proteins are imported from the cytoplasm into the mitochondria (Fig. 1, panels C and D). No changes were observed in the cytoplasmic amounts of RNase L, RLI, or GAPDH after CCCP treatment.

The presence of RNase L in mitochondria was also established by immunofluorescence and confocal microscopy. A fraction of the cellular RNase L population clearly co-localized with MitoTracker, a mitochondria-specific marker (30–32). The yellow coloration (Fig. 2, panel C) resulting from the combined fluorescence of MitoTracker (red fluorescence, Fig. 2, panel A) and fluorescein linked to a secondary antibody against the rabbit anti-RNase L polyclonal antibody (green fluorescence, Fig. 2, panel B) clearly showed this co-localization.

Mitochondrial mRNAs Are Targets of RNase L—Because RNase L was present in the mitochondria, we proceeded with pulse-chase experiments on intact mitochondria isolated from H9 cells to confirm that mitochondrial mRNAs could be destabilized by mitochondrial RNase L. We followed the stability of radiolabeled mitochondrial mRNAs in the presence or not of 2-5A, the specific activator of RNase L (10, 13). As illustrated by the data in Fig. 3, the addition of 2-5A to isolated intact mitochondria destabilizes mitochondrial mRNAs including cytochrome b, ATPase 6, and cytochrome oxidase subunit II. This result clearly shows that mitochondrial RNase L, once activated directly by 2-5A, can destabilize mitochondrial mRNAs.

Decreased RNase L Activity Inhibits IFNα-induced Down-regulation of Mitochondrial mRNAs—To study the potential role of RNase L and RLI in the down-regulation of mitochondrial mRNAs by IFNα, we reduced endogenous RNase L activity in human H9 T lymphocytes, chosen for their sensitivity to the antiproliferative effect of IFNα, by stably transfecting an RNase L cDNA antisense construct or an RLI sense cDNA construct. Two clones from each stable transfection were selected based on their decreased 2-5A binding capacity, as an indicator of diminished cellular RNase L activity and their RLI and RNase L protein content (Fig. 4). The two RLI sense cDNA
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Fig. 4. Characterization of the H9 transfectants. A, post-nuclear extracts (100 μg of proteins) from H9 cells transfected with the empty vector (control), the RLI sense cDNA (RLI S1 and RLI S2), or the RNase L cDNA in the antisense orientation (RNase L AS1 and RNase L AS2) were analyzed by the radioisotopic affinity labeling assay (2–5ApCp) for RNase L 2–5A binding activity or by Western blot with the appropriate polyclonal antibodies for RNase L, RLI, and GAPDH proteins. B, densitometric analysis of the gels shown in panel A. A value of 100% corresponds to the amount of RNase L 2–5A binding activity (2–5ApCp) or RNase L and RLI protein in control cells. Error bars refer to the S.D. obtained in three independent experiments.

transfected clones (RLI S1 and RLI S2) expressed the same level of RNase L compared with the control cells but exhibited a 2-fold decrease in 2–5A binding activity due to RLI overexpression (Fig. 4, panels A and B). In these two clones, there was a strong correlation between RLI overexpression and down-regulation of RNase L 2–5A binding activity. In the two RNase L antisense cDNA transfectant clones (RNase L AS1 and RNase L AS2), decreased 2–5A binding activity correlated with lower expression levels of RNase L and no change in RLI expression levels. The level of GAPDH protein was constant in control and in transfected cells.

These clones were treated with IFNα (500 units/ml) for 24 and 48 h, and their mitochondrial mRNAs levels were analyzed by Northern blot (Fig. 5). In the control H9 cells, IFNα treatment down-regulated several mitochondrial mRNAs such as cytochrome b, ATPase 6, and cytochrome oxidase subunit II, as previously observed in other cell types (7, 8, 33). In contrast, in the RLI S and RNase L AS transfectants, no decrease in these mitochondrial mRNAs was observed after IFNα treatment. The 6–16 mRNA, a known IFNα-induced gene, was correctly induced by IFNα treatment in all transfectants as in control cells and as previously described (34), indicating that these cells responded to IFNα treatment. The level of 18 S rRNA was constant in control and in transfected cells. These experiments thus support a role of RNase L in the accelerated degradation of mitochondrial mRNAs in IFNα-treated cells.

Inhibition of the Antiproliferative Effect of IFNα in H9 Transfectants—IFNα exerts an antiproliferative effect on various cell lines, and the down-regulation of mitochondrial mRNAs is only observed in cells sensitive to the antiproliferative effect of IFNα.

There is a correlation between the antiproliferative response and the mitochondrial response (7, 8, 33). This correlation and the fact that a decrease in mitochondrial mRNAs leads to a decrease in mitochondrial proteins and to a decrease in cellular ATP levels (7, 8) suggests that the down-regulation of mitochondrial mRNAs could participate in the growth inhibition induced by IFNα. Therefore, we charted the growth rate of H9 cells and H9 transfectants for 5 days after IFNα (500 units/ml) treatment. IFNα inhibited cell proliferation in the control H9 cells, transfected with an empty vector (Fig. 6, panel A). On the contrary, the H9 transfectants with no IFNα-induced down-regulation of mitochondrial mRNAs (Fig. 5) were resistant to the growth inhibitory effects of IFNα (Fig. 6, panels B–E).

No Mitochondrial mRNAs Destabilization Is Observed after IFNα Treatment when Mitochondrial RNase L Activity Is Low—To determine whether there is a correlation between the down-regulation of mitochondrial mRNAs and RNase L activity in the mitochondria, we performed a radiolabeled affinity labeling assay on purified mitochondrial extracts from the H9 transfectants. Less 2–5A binding activity of RNase L was detected in the mitochondria of the H9 transfectants than in the control H9 cells (Fig. 7, panels A and B). In the RLI S1 and RLI S2 clones, the decreased RNase L 2–5A binding activity correlated with an increased level of RLI in the mitochondria. In the RNase L AS1 and RNase L AS2 clones, the quantity of RLI found in mitochondria was the same as that in the control cells, indicating that the decrease of RNase L 2–5A binding activity is probably because of low levels of RNase L, as shown for total cell extract (Fig. 4). The absence of GAPDH confirms that there was no cytoplasmic contamination in the mitochondrial extract, and MRP12 levels indicate that an equal amount of mitochondrial proteins was loaded for each transfectant.

Because our data demonstrated that the 2–5A/RNase L/RLI pathway is involved in the down-regulation of mitochondrial mRNAs by IFNα (Fig. 5), that RNase L was present in the mitochondria, and that mitochondrial mRNAs can be destabilized by mitochondrial RNase L after its activation by 2–5A (Figs. 1–3), we postulated that mitochondrial RNase L may regulate mitochondrial mRNA stability in response to IFNα treatment. To verify this hypothesis, we compared the stability of radiolabeled mitochondrial mRNAs by pulse-chase analysis of intact mitochondria isolated from control H9 cells and from RLI S- and RNase L AS-transfected H9 cells treated or not with IFNα. As shown in Fig. 8, IFNα treatment of control H9 cells resulted in the destabilization of mitochondrial mRNAs cytochrome b, ATPase 6, and cytochrome oxidase subunit II. It is worth noting that the steady state level of radiolabeled mitochondrial mRNAs obtained in control IFNα-treated cells was decreased by 30% (compare times 0 on autoradiographies in Fig. 8). Because these radiolabeled mitochondrial mRNAs are obtained by transcription in mitochondria isolated from control IFNα-treated cells, this could indicate that IFNα treatment...
also decreased mitochondrial mRNAs transcription in control cells, as suggested by Lou et al. (7). This can also be explained either as a destabilization of the mitochondrial mRNA by the activated RNase L in the IFNα/H9251-treated cells simultaneous with transcription or as a combination of a decreased transcription and degradation of mitochondrial mRNA. Our results, showing a destabilization of mitochondrial mRNAs in control IFNα/H9251-treated cells, are in favor of the second and third hypotheses. In the RLI S1 and RNase L AS2 H9 transfectants, with low RNase L activity, the mitochondrial mRNAs remained stable upon IFNα treatment (Fig. 9; panels A and B).

**DISCUSSION**

We were able to detect RNase L and its inhibitor, RLI, in isolated mitochondria (Figs. 1, 2, and 7) and showed that mitochondrial RNase L could destabilize mitochondrial mRNAs after its activation by its specific activator, 2′−5′A, in intact mitochondria (Fig. 3). These results presented the possibility that RNase L may degrade mitochondrial mRNAs after IFNα treatment. Furthermore, previous reports describe the presence of the p69 isoform of 2′−5′A synthetase in mitochondria (35, 36). IFNα treatment increases the RNase L level in mitochondria (Fig. 1) as it was shown for 2′−5′A synthetase (35). It was therefore conceivable that IFNα treatment could activate the 2′−5′A/RNase L pathway in mitochondria and promote the destabilization of mitochondrial mRNAs, as described here. RNase L and RLI are imported from the cytoplasm into the mitochondria because suppression of the mitochondrial membrane potential by CCCP diminished RNase L and RLI mitochondrial level (Fig. 1). The import of proteins into mitochondria is a complex mechanism that is not completely understood. In fact not all the mitochondrial-targeted proteins obey the same rule. The majority of mitochondrial proteins are nuclear-encoded and synthesized in the cytosol as precursors proteins. These proteins are imported into mitochondria by a complex process that involves cytosolic chaperones, an hetero-oligomeric translocase of the outer mitochondrial membrane (TOM) complex, a translocase complex in the inner membrane (TIM), an ATP-driven import motor associated with TIM and soluble matrix proteins mediating proteolytic maturation and folding of the imported proteins (37–39). Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the β-subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein. Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the β-subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein. Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the β-subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein. Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the β-subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein. Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the β-subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein. Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the β-subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein.
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Polyacrylamide gel electrophoresis, indicating that it is not processed after import into the mitochondria (Figs. 1 and 7). RLI possesses an uncevable mitochondrial N-terminal-targeting sequence (an amphiphilic α-helix), and the mitochondrial membrane potential is required for its import because CCCP treatment of H9 cells decreased the level of RLI in mitochondria (Fig. 1). One hypothesis is that RLI is imported into the mitochondria via the TOM-TIM pathway (38, 39), but this remains to be demonstrated experimentally.

Because secondary structure analysis does not predict the presence of an amphiphilic α-helix at the amino terminus of RNase L, we analyzed the RNase L sequence using PSORT II, a program used to predict protein localization (51–53). This program is based on data obtained experimentally (54). Such analysis of RNase L amino acid sequence gives the highest score (39%) for a mitochondrial localization of RNase L. Our experimental results and computer analysis together indicate that RNase L has a mitochondrial localization but does not possess an amino-terminal-targeting sequence. These results indicate that the mitochondrial-targeting sequence information of RNase L is internal, as has been described for other proteins as apocytochrome c or p53. The mechanisms of recognition and transport of such proteins are unknown or only partially understood (38, 43).

To study the role of the 2′–5′A/RNase L pathway and its inhibitor, RLI, in the IFN-α-induced down-regulation of mitochondrial mRNAs, we down-regulated RNase L activity in human H9 T lymphocytes by stably transfecting (i) RNase L antisense (RNase L AS) cDNA, which inhibits RNase L expression and consequently RNase L activity, or (ii) RLI sense (RLI S) cDNA (Figs. 4 and 7). In contrast to mock-transfected control cells, in the transfectants with decreased RNase L activity, we did not observe a down-regulation of mitochondrial mRNAs by IFNα (Fig. 5). These results revealed a direct correlation between mitochondrial mRNAs decrease and the level of RNase L activity in the mitochondria (Figs. 5 and 7).

We followed the stability of radioabeled mitochondrial mRNAs using pulse-chase analysis in intact mitochondria isolated from H9 transfectants treated or not with IFNα. IFNα treatment clearly induced a diminution of the stability of mitochondrial mRNAs in the control H9 cells (Fig. 8). These mitochondrial mRNAs remained stable in the H9 transfectants with decreased mitochondrial RNase L activity (Fig. 9). This result indicates that mitochondrial RNase L participates in mitochondrial mRNAs regulation in IFNα-treated cells (Figs. 5, 7, 8, and 9). Interestingly, the growth inhibition activity of IFNα is abolished in parallel with the stabilization of mitochondrial mRNAs (Figs. 5 and 6). This result confirms and extends the previous observations that the down-regulation of mitochondrial mRNAs by IFNα was observed only in cells sensitive to the antiproliferative effect of IFN (7, 8).

rRNA and viral RNAs have been the first described targets of RNase L. Recently, cellular mRNAs have been identified as regulated by RNase L. ISG43 and ISG15 mRNAs were shown to be destabilized by RNase L after IFN treatment (55), and we identified MyoD mRNA as a target of RNase L during C2 myoblast differentiation (56). Here, we demonstrate that mitochondrial mRNAs decrease during IFNα treatment because of post-transcriptional events in which RNase L plays a central role. These data provide new and direct evidence for a more general role of RNase L in the regulation of cellular mRNAs. The demonstration that mitochondrial mRNAs are targets for RNase L may explain how RNase L and RLI could regulate the antiproliferative effect of IFN. Moreover, these results could explain how RNase L plays a role in apoptosis. Indeed, mitochondria play a central role in apoptosis by releasing cyto-

**Fig. 6. Antiproliferative activity of IFNα on H9-transfected cells.** H9-transfected cells were treated by IFNα (500 units/ml) (●) or not (□), and cell growth was monitored each day by counting viable cells as determined by trypan blue exclusion. A, H9 cells transfected with the empty vector (control). B and C, H9 cells transfected with the RLI sense cDNA (RLI S1 and RLI S2 respectively). D and E, H9 cells transfected with the RNase L antisense cDNA orientation (RNase L AS1 and RNase L AS2, respectively). A value of 100% corresponds to the number of control or transfected cells at day 5 and untreated by IFNα. Error bars refer to the S.D. obtained in three independent experiments.
chrome c, which leads to the activation of caspase-3 (57–60), and several studies implicate RNase L in apoptosis (61–64). Degradation of mitochondrial mRNAs by RNase L will obviously lead to inhibition of mitochondrial protein synthesis, as Lewis et al. (8) show that decrease in mitochondrial mRNA levels leads to a decrease in mitochondrial protein levels. The consequence of such an inhibition is a loss of mitochondrial membrane potential, which results in osmotic swelling and

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**Fig. 7.** RNase L and RLI analysis in mitochondria of H9 transfectants. A, mitochondrial extracts (200 μg of protein) from H9 cells transfected with the empty vector (control), with the RLI sense construction (RLI S1 and RLI S2), and with the RNase L antisense construction (RNase L AS1 and RNase L AS2) were analyzed by the radiocovalent affinity-labeling assay (2–5ApCp) for RNase L 2–5A binding activity and by Western blot with the appropriate polyclonal antibodies for RLI, MRLP12, and GAPDH protein contents. B, densitometric analysis of the gels shown in panel A. A value of 100% corresponds to the amount of proteins in mitochondrial extracts of control cells. Error bars refer to the S.D. obtained in three independent experiments.

**Fig. 8.** Mitochondrial mRNAs are destabilized in control H9 cells treated by IFNα. Control H9 cells transfected with the empty vector were treated by IFNα (+) or not (−) for 24 h. Mitochondria were isolated from these cells, and radioactive transcription was performed with [α-32P]UTP for 1 h at 37 °C followed by a chase with unlabeled UTP. At the indicated times, mitochondrial RNAs were extracted and hybridized with spotted mitochondrial cytochrome b cDNA (CYTB) (A), ATPase 6 cDNA (ATP6) (B), cytochrome oxidase II cDNA (CO) (C). A densitometric analysis of the gels is shown at the right side of each autoradiography. Data are presented as the log of RNA remaining versus time. A value of 1 corresponds to the log of the amount of RNA at time 0, before incubation with unlabeled UTP in control H9 cells treated (+) or not (−) by IFNα. The S.D. obtained from three independent experiments are indicated by error bars.
cytochrome c release (58). In the same way, Yanase et al. (65) have recently shown that during IFN/H9251-induced apoptosis there is a release of cytochrome c from mitochondria, a decline in mitochondrial membrane potential, and caspase-3 activation (65). On the other hand, activation of RNase L causes caspase-dependent apoptosis accompanied by cytochrome c release (66), (67). This induction of cell death by RNase L is inhibited by Bcl-2 (66). These results and our results show that the regulation of mitochondrial mRNAs by RNase L and its inhibitor RLI is at the center of the pathway leading from IFN/H9251 to apoptosis.

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REFERENCES

Fig. 9. Mitochondrial mRNAs are stable in RLI S1 and RNase L AS2 H9 transfec-tants treated by IFNa. H9 cells transfected with the RLI sense construction (RLI S1) or with the RNase L antisense construction (RNase L AS2) were treated (+) or not (−) by IFNa for 24 h. Pulse-chase experiments were performed on mitochondria isolated from RLI S1 (A) and RNase L AS2 (B) H9 transfectants and analyzed as described in the legend of Fig. 8. A densitometric analysis of the gels is shown under each autoradiography. Data are presented as the log of RNA remaining versus time. A value of 1 corresponds to the log of the amount of RNA at time 0, before incubation with unlabeled UTP in each transfected cells treated (■) or not (□) by IFNa. The S.D. obtained from three independent experiments are indicated by error bars. CYTB, cytochrome b; ATP6, ATPase 6; COII, cytochrome oxidase subunit II.
Additions and Corrections


Control of prostaglandin stereochemistry at the 15-carbon by cyclooxygenases-1 and -2. A critical role for serine 530 and valine 349.

Claus Schneider, William E. Boeglin, Jeffery J. Prusakiewicz, Scott W. Rowlinson, Lawrence J. Marnett, Nigulas Samel, and Alan R. Brash

Page 481, Table I: Several columns in this table were misaligned. The correctly aligned table is shown following.

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The 2-5A/RNase L/RNase L inhibitor (RLI) pathway regulates mitochondrial mRNAs stability in interferon α-treated H9 cells.

Florence Le Roy, Catherine Bisbal, Michelle Silhol, Camille Martinand, Bernard Lebleu, and Tamim Salehzada

The abbreviation within the title was printed incorrectly. The correct title is shown above.

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