Cell Death and Differentiation (2007) 14, 1406–1413

www.nature.com/cdd

Regulation of mitochondrial mRNA stability by RNase L is translation-dependent and controls IFNα-induced apoptosis

F Le Roy*[,1,3], M Silhol[1,4], T Salehzada[2] and C Bisbal*[,2]

Interferons (IFNs) inhibit the growth of many different cell types by altering the expression of specific genes. IFNs activities are partly mediated by the 2′-5′ oligoadenylates-RNase L RNA decay pathway. RNase L is an endoribonuclease requiring activation by 2′-5′ oligoadenylates to cleave single-stranded RNA. Here, we present evidence that degradation of mitochondrial mRNA by RNase L leads to cytochrome *c* release and caspase 3 activation during IFNα-induced apoptosis. We identify and characterize the mitochondrial translation initiation factor (IF2mt) as a new partner of RNase L. Moreover, we show that specific inhibition of mitochondrial translation with chloramphenicol inhibits the IFNα-induced degradation of mitochondrial mRNA by RNase L. Finally, we demonstrate that overexpression of IF2mt in human H9 cells stabilizes mitochondrial mRNA, inhibits apoptosis induced by IFNα and partially reverses IFNα-cell growth inhibition. On the basis of our results, we propose a model describing how RNase L regulates mitochondrial mRNA stability through its interaction with IF2mt.
Cell Death and Differentiation (2007) **14,** 1406–1413; doi:10.1038/sj.cdd.4402130; published online 13 April 2007

Apoptosis is a regulated cell death process that plays a central role in the control of many physiological events. Cells die by apoptosis during embryonic development, tissue homeostasis or immune regulation and defects in the apoptotic pathway during these events can lead to excessive cell accumulation with dramatic consequences.[1–3] Interferons (IFNs) are among the regulators of apoptosis. They belong to a family of cytokines produced and secreted by mammalian cells in response to various inducers. They are negative regulators of cell proliferation through induction of cell-cycle arrest and apoptosis, but the mechanisms are not yet fully understood. The 2–5A/RNase L pathway is a single-stranded RNA (ssRNA) decay pathway induced by IFNs: the 2–5A synthetases are induced by IFNs and upon activation by double-stranded RNA (dsRNA), convert ATP into a series of oligomers known as 2′-5′ oligoadenylates (2–5A).[4,5] The 2–5A activates RNase L, a latent endoribonuclease, which inhibits protein synthesis by cleaving ssRNA.[6,7]

RNase L plays a central role in IFNs cell growth inhibition and in IFN-induced apoptosis.[8–12] Activation of RNase L causes caspase-dependent apoptosis accompanied by cytochrome *c* release from the mitochondria.[13–15] Moreover, we have shown that part of RNase L is localized in mitochondria and regulates mitochondrial mRNA stability in IFNα-treated cells.[12]

RNase L is an endoribonuclease which has little sequence specificity as it cleaves RNA 3′ of UpNp nucleotides.[6,7] On the other hand, only a few cellular RNAs have been identified as regulated by RNase L: rRNA,[16] IFN-induced genes ISG43 and ISG15 mRNAs,[17] mitochondrial mRNAs,[12,18] MyoD mRNA during C2 myoblast differentiation[19] and PKR mRNA.[20]

RNase L activity is regulated not only by 2–5A binding, but also by interaction with other proteins. It has been shown previously that RNase L can form a heterodimer with the RNase L inhibitor (RLI), a protein that inhibits 2–5A binding to RNase L, resulting in an inhibition of RNase L activity.[21,22] More recently, we demonstrated that RNase L interacts with the translation termination release factor eRF3/GSPT1 and that their interaction is important for its role in translation termination regulation.[23]

We set out to identify other potential RNase L partners to better comprehend its mechanism of action. In the present study, a yeast two-hybrid screening, using human RNase L as bait identifies the mitochondrial initiation factor IF2mt as a partner of RNase L. We demonstrate that RNase L interacts with IF2mt *in vitro* and that this interaction modulates mitochondrial mRNAs stability *in vivo*. Moreover, overexpression of IF2mt abrogates mitochondrial RNase L activity, inhibits IFNα-induced apoptosis and partially reverses

[1]UMR 5535, Institut de Génétique Moléculaire de Montpellier, 1919 route de Mende, 349293 Montpellier, France and [2]UPR CNRS 1142, Institut de Génétique Humaine, 141 rue de la Cardonille, 34396 Montpellier, France
*Corresponding authors: C Bisbal, UPR CNRS 1142, Institut de Génétique Humaine, 141 rue de la Cardonille, 34396 Montpellier, France. Tel: +330 499 619 973; Fax: +330 499 619 901; E-mail: Catherine.Bisbal@igh.cnrs.fr (and) F Le Roy, CNRS FRE2937, Génétique Moléculaire et Intégration des Fonctions Cellulaires, Institut André Lwoff, 7 rue Guy Moquet, BP3 94801 Villejuif Cedex, France. Tel: +33 01 49 58 33 80; Fax: +33 01 49 58 33 81; E-mail: leroyfg@vjf.cnrs.fr
[3]Present address: CNRS FRE2937, Génétique Moléculaire et Intégration des Fonctions Cellulaires, Institut André Lwoff, 7 rue Guy Moquet, BP3 94801 Villejuif Cedex, France.
[4]Present address: Mécanismes moléculaires dans les démences neurodégénératives, INSERM 710, Place Eugéne Bataillon, 34095 Montpellier, France.
Keywords: apoptosis; mitochondrial mRNA; IFNα; mRNA stability; RNase L
Abbreviations: IFNα2, interferon α2; RNase L, endoribonuclease L; RLI, Ribonuclease L Inhibitor; 2-5A, 2′-5′-oligoadenylate; OAS1, 2′-5′-oligoadenylate synthetase (40/46 kDa); OAS2, 2′-5′-oligoadenylate synthetase (69/71 kDa); CYTB, cytochrome *b*; ATP 6, ATPase 6; CAM, chloramphenicol; NP-40, Nonidet P-40; PMSF, phenyl methyl sulfonyl fluoride; DTT, dithiothreitol; IF2mt, mitochondrial translation initiation factor 2
Received 16.8.06; revised 15.2.07; accepted 26.2.07; Edited by JA Trapani; published online 13.4.07

antiproliferative activity of IFNα. These results outline a new mechanism for RNase L as a regulator of mitochondrial mRNA stability through its recruitment to mRNA during their translation via its interaction with IF2mt.

Results

RNase L interacts with IF2mt. To identify potential RNase L partners and better understand its mechanism of action, we performed a yeast two-hybrid screening using the human RNase L as bait and a human HeLa S3 cDNA library as a prey. One of the positive clones was identified by sequence analysis as the mitochondrial translation initiation factor 2 (IF2mt).[24] IF2mt promotes the binding of fMet-tRNA to the small subunit of mitochondrial ribosomes.[25] IF2mt is encoded by a precursor of 90 kDa, which, following its import in the mitochondria, is cleaved to give rise to an 85 kDa mature protein. The identification of IF2mt as a partner of RNase L is consistent with the previously observed mitochondrial localization of RNase L in H9 and HeLa cells.[12]

We confirmed the interaction between RNase L and IF2mt by pull-down and co-immunoprecipitation assays**.** For the pull-down assay, RNase L was tagged with GST in the C terminus and IF2mt was translated in the presence of radioactive methionine in rabbit reticulocyte lysate (RRL). The recombinant proteins, RNase L-GST or GST, were bound to glutathione-sepharose and mixed with RRL containing the radiolabeled IF2mt. The resulting bound and unbound proteins to glutathione-sepharose were analyzed by SDS-PAGE (Figure 1a) and the radiolabeled IF2mt associated with the beads was quantified (Figure 1b). The results show that IF2mt coprecipitates specifically with RNase L-GST (Figure 1a, lane 4), but is very weakly associated with GST (Figure 1a, compare lanes 3 and 4). The interaction observed between IF2mt and RNase L is significant as the quantification reveals that 30% of the input radiolabeled IF2mt is bound to RNase L-GST compared to only 2% to GST (Figure 1b, compare lane 4 with 2 and lane 3 with 1). For the co-immunoprecipitation assay, we incubated RRL containing the radiolabeled IF2mt with a polyclonal antibody directed against RNase L [12] or the nonspecific LexA antibody and protein A-sepharose. The radiolabeled proteins associated with the beads were analyzed by SDS-PAGE (Figure 1c). The protein IF2mt immunoprecipitates with RNase L antibody and not with the nonspecific LexA antibody (Figure 1c, compare lanes 2 and 3). The input (10%) radiolabeled IF2mt is represented in lane 1. Taken together, these results show that RNase L binds IF2mt.

Decrease of mitochondrial mRNA stability by IFNα2 is translation dependent. As IF2mt is a mitochondrial translation initiation factor and RNase L has been shown previously to regulate mitochondrial mRNA stability,[12] we tested whether an active translation was required for the destabilization of mitochondrial mRNA by IFNα-activated RNase L. To address this question, we measured mitochondrial mRNA level in H9 cells treated with IFNα2 and/or chloramphenicol (CAM) to inhibit mitochondrial translation (Figure 2).[26] The H9 cells treated with IFNα2 show a decreased level of ATPase 6 (ATP6), cytochrome *b* (CYTB) and cytochrome oxidase II (COII) mRNAs compared to the untreated cells (Figure 2a, compare lanes 1 and 2) as previously observed.[12] On the contrary, the cells treated with IFNα2 and CAM show the same level of ATP6, CYTB and COII mRNAs as untreated cells or CAM-treated cells (Figure 2a, compare lane 4 to 1 and 3). As a positive control of IFNα2 activity, we used the 6–16 nuclear gene which is induced transcriptionally by IFNα2 (Figure 2a, compare lane 1 with 2 and 3 with 4).[27] These results demonstrate that inhibition of mitochondrial translation

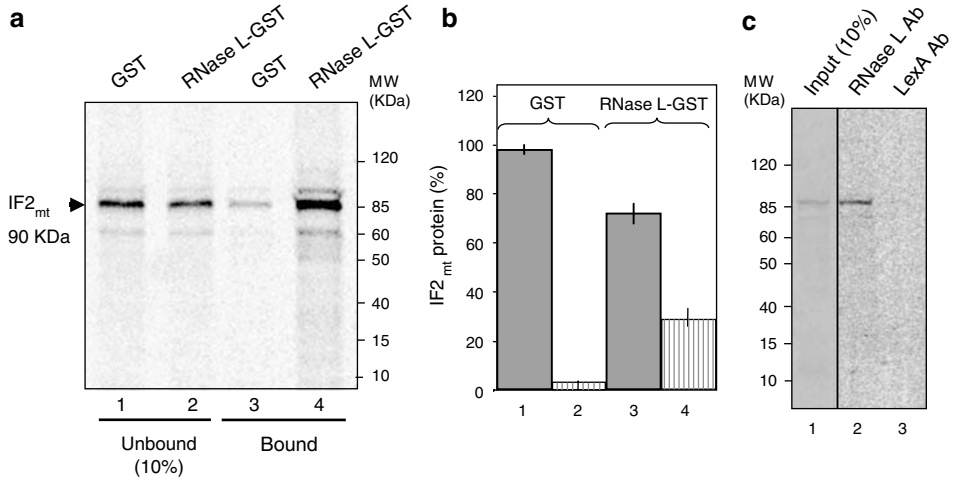

Figure 1 RNase L interacts with IF2mt. (**a**) IF2mt was translated in the presence of S^{35}-methionine in RRL and mixed with recombinant RNase L-GST or GST proteins bound to glutathione-sepharose. The unbound proteins (10% of the fraction) and the proteins bound to the beads were analyzed by SDS-PAGE and PhosphorImager. (**b**) Quantification of the S^{35}-radiolabeled IF2mt unbound to GST (lane 1), bound to GST (lane 2), unbound to RNaseL-GST (lane 3) and bound to RNase L-GST (lane 4) (Imagequant software). (**c**) IF2mt was translated in the presence of S^{35}-methionine in RRL. RRL containing S^{35}-radiolabeled IF2mt was incubated with a polyclonal antibody directed against RNase L (lane 2) or Lex A (lane 3) and protein A-sepharose. The radiolabeled proteins associated with the beads were analyzed by SDS-PAGE and PhosphorImager. RRL containing S^{35}-radiolabeled IF2mt (10%) was added as a control (lane 1)

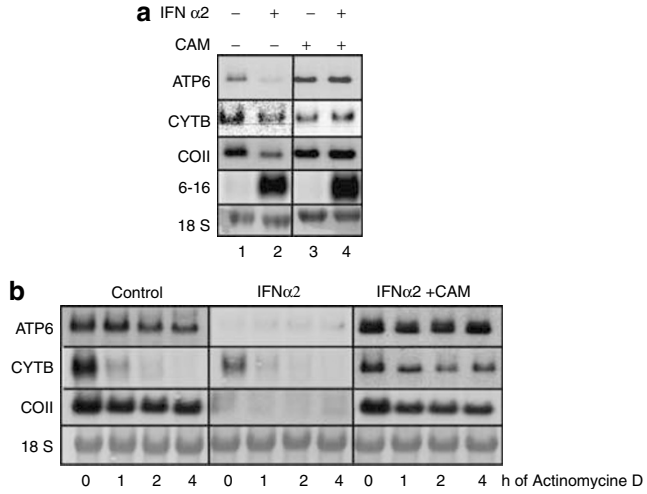

Figure 2 Decrease of mitochondrial mRNA stability by IFNα2 is dependent on translation. (**a**) Northern blot analysis of mitochondrial mRNAs (ATP6, CYTB and COII) and 6-16 nuclear-encoded mRNA from H9 cells treated with IFNα2 (lane 2), chloramphenicol (CAM) (lane 3), CAM and IFNα2 (lane 4) or untreated (lane 1). (**b**) Northern blot analysis of mitochondrial mRNAs (ATP6, CYTB and COII) from H9 cells treated with Actinomycin D up to 4 h. These cells were simultaneously treated with IFNα2 (IFNα2), IFNα2 and chloramphenicol (IFNα2 + CAM) or untreated (Control). Coloration of 18S RNA was used as indicator of equal RNA loading between the different gel lanes

blocks mitochondrial mRNA downregulation induced by IFNα2.

To confirm that the mitochondrial mRNA decrease observed during the IFNα2 treatment (Figure 2a) is due to mRNA decay, we compared their stability following actinomycin D chase (Figure 2b).[26] We observe a destabilization of mitochondrial mRNAs CYTb, ATP6 and COII when H9 cells are treated with IFNα2 (Figure 2b, compare IFNα2 with Control). On the contrary, we observe a stabilization of mitochondrial mRNAs in H9 cells treated with IFNα2 and chloramphenicol compared to the cells treated with IFNα2 alone (Figure 2b). These results suggest that RNase L regulates mitochondrial mRNA stability in a translation-dependent manner.

RNase L/IF2mt interaction modulates RNase L activity. To confirm the role of the RNase L/IF2mt interaction in the translation-dependent decay of mitochondrial mRNAs, we overexpressed IF2mt in H9 cells. We hypothesized that an excess level of IF2mt protein will trap RNase L away from the mitochondrial translation initiation complex and will inhibit mitochondrial mRNA degradation. We stably transfected H9 cells with a plasmid encoding IF2mt. As no antibody against human IF2mt is available, we selected the clone expressing the highest level of IF2mt mRNA (IF2mt2) for the following experiments (Figure 3a, IF2mt endo + transf, compare lane 3 with 1 and 2, Figure 3b). We also controlled the levels of 2′-5′-oligoadenylate synthetases (OAS1, OAS2), RNase L and RLI mRNAs. These mRNAs are expressed at the same level as in the parental cells (compare lanes 2 and 3 to 1). We tested IF2mt2 clone for its response to IFN and monitored the induction of the OAS1, OAS2 and RNase L mRNAs after 24 h of IFNα2 treatment (Figure 3c, compare lanes 2 and 1, Figure 3d). As in parental cells (Figure 3c, lanes 3 and 4), we observe a 1.5-fold increase in RNase L and OAS2 mRNAs level, a fourfold increase in OAS1 mRNA level following IFNα2 treatment compared to eIF1α mRNA, which is not regulated by IFN (Figure 3c, compare lanes 2 and 1 and Figure 3d). Interestingly, IF2mt mRNA level, like RLI, is not regulated by IFNα2 in H9 cells (Figure 3c, compare lanes 2 and 1; Figure 3d).

In IF2mt2 clone treated by IFNα2, we observe a stabilization of CYTB, ATP6 and COII mRNA level compared to the parental cells (Figure 4, compare IF2mt with control). Similar results are observed in cells with decreased RNase L activity owing to the expression of RNase L antisense (Figure 4, compare IF2mt with RNaseLAS).[12] These results show that overexpression of IF2mt blocks RNase L nuclease activity induced by IFNα2 in mitochondria.

We have shown previously that the downregulation of the mitochondrial mRNAs by RNase L is observed during the antiproliferative effect of IFNα2.[12] To study the role of the RNase L/IF2mt interaction in the antiproliferative effect of IFNα2, we measure the growth rate of H9 cells overexpressing IF2mt (IF2mt2 clone). We observe an 80% inhibition of control H9 cells growth after IFNα2 treatment (Figure 5a, open square compared to closed square). On the other hand, IF2mt2 cells treated with IFNα2 show a 60% growth inhibition compared to untreated IF2mt2 cells (Figure 5a, open triangle compared to closed triangle). In contrast, downregulation of RNase L activity by RNase L antisense completely reverses IFNα2-induced inhibition of cell proliferation (Figure 5b, open square compared to closed square). Our results demonstrate that IF2mt overexpression only partially reverses antiproliferative activity of IFNα observed in control H9 cells.

IFNα-induced apoptosis is associated with cytochrome *c* release in the cytoplasm and caspase 3 activation.[28,29] RNase L plays an important role in IFNα-induced apoptosis[9,10] and its activation causes caspase-dependent apoptosis accompanied by cytochrome *c* release from the mitochondria.[13–15] To investigate the role of the RNase L/IF2mt interaction in IFNα-induced apoptosis, we measured cytochrome *c* release, caspase 3 cleavage and its activity in H9 cells overexpressing IF2mt (IF2mt2 clone). In H9 control cells, cytochrome *c* release and caspase 3 cleavage are induced after 24 and 48 h treatment with IFNα2 (Figure 6a, lanes 2 and 3 compared with lane 1, Figure 6d). On the contrary, we do not observe cytochrome *c* release in IF2mt2 cells treated with IFNα2 for 24 and 48 h (Figure 6a, compare lanes 5 and 6 with lanes 2 and 3, Figure 6c) or caspase 3 cleavage in IF2mt2 cells treated with IFNα2 for 24 h (Figure 6a, compare lane 5 with lanes 2 and 4, Figure 6d). After 48 h of IFNα2 treatment, we can observe a small amount of caspase 3 cleavage products, p17 and p15, in IF2mt2 cells, but close to the background level observed in IF2mt2 cells not treated with IFNα2 (Figure 6a, compare lanes 6 and 4). The amount of cleaved caspase 3 (10%) in IF2mt cells is lower than in H9 control cells, where nearly all the p35 caspase 3 (85%) has been cleaved (Figure 6a, compare lane 6 with lane 3, Figure 6c,d). Equal protein loading between the different cell extracts was monitored with polyclonal antibodies against GAPDH (Figure 6a). Moreover, caspase 3

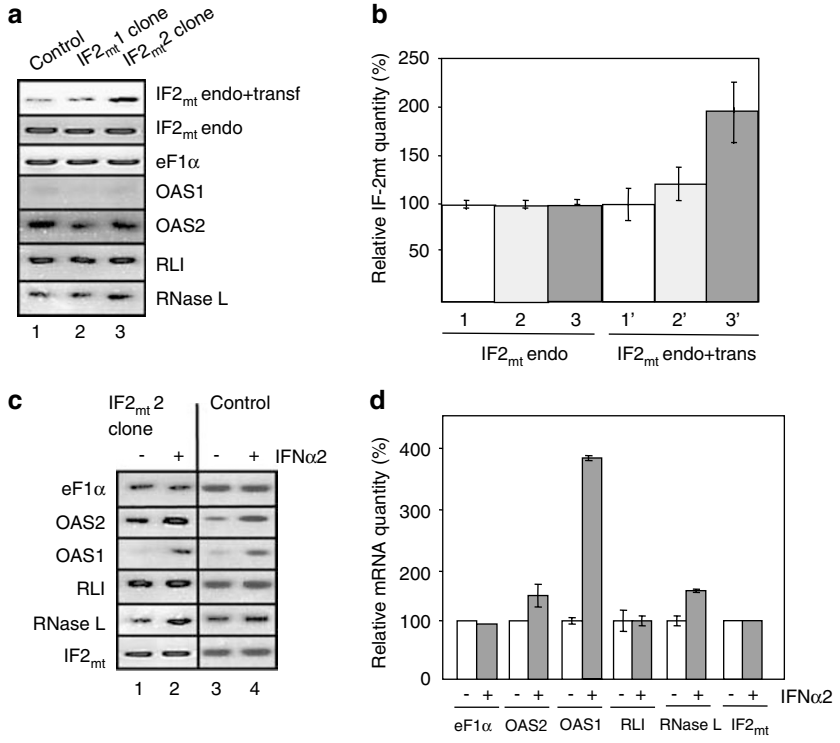

Figure 3 Characterization of H9 clones overexpressing IF2mt. (**a**) Semiquantitative PCR analysis of eF1α, OAS1, OAS2, RLI, RNase L mRNAs, endogenous IF2mt mRNA (IF2mt endo), endogenous and transfected IF2mt mRNAs (IF2mt endo + transf) in H9 cells control (lane 1), IF2mt1 clone (lane 2) and IF2mt2 clone (lane 3). (**b**) Quantification of endogenous IF2mt in control cells (lane 1), IF2mt1 clone (lane 2), IF2mt2 clone (lane 3) and of endogenous and transfected IF2mt in control cells (lane 1′) IF2mt1 clone (lane 2′), IF2mt2 clone (lane 3′). (**c**) Semiquantitative PCR analysis of eF1α, OAS1, OAS2, RLI, RNase L, IF2mt mRNAs in H9 cells overexpressing IF2mt (IF2mt2) untreated (lane 1) or treated with IFNα2 for 24 h (lane 2) and in H9 control cells untreated (lane 3) or treated with IFNα2 for 24 h (lane 4). (**d**) Quantification of eF1α, OAS1, OAS2, RLI, RNase L and IF2mt mRNA in IF2mt2 clone treated (+) or not (−) with IFNα2 for 24 h. A value of 100% corresponds to the quantity of the different mRNA in control cells. Quantification of eF1α mRNA was used as a control of equal cDNA quantity used in each semiquantitative PCR. The experiments were done in triplicate and the standard deviations are indicated on the plots

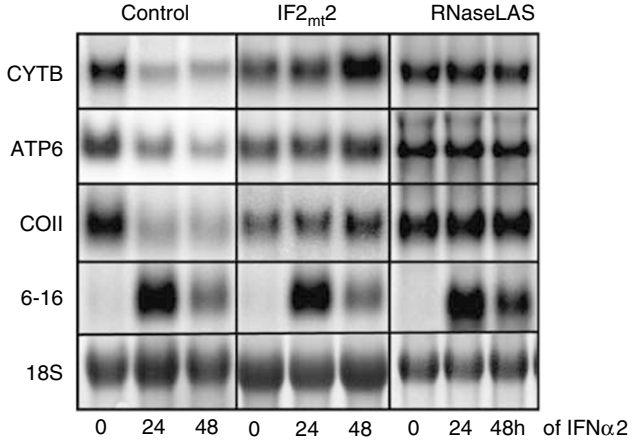

Figure 4 IF2mt overexpression stabilizes mitochondrial mRNA in H9 cells treated with IFNα2. Northern blot analysis of mitochondrial mRNA (CYTB, ATP6 and COII) from H9 cells overexpressing IF2mt (IF2mt2), RNase L antisense (RNaseLAS) or not (control) treated with IFNα2 up to 48 h. Transcriptional induction of 6–16 nuclear gene is a positive control of IFNα2 activity. Coloration of 18S RNA was used as control of equal RNA loading between the different gel lanes

cleavage is accompanied by caspase 3 activity in control H9 cells treated with IFNα2, but not in IF2mt2 cells treated with IFNα2 (Figure 6e). These observations are not due to variation of RNase L or RLI protein level in the IF2mt2 cells compared with control cells (Figure 6a, compare lanes 2 and 3 with 5 and 6, Figure 6b). These results demonstrate that overexpression of IF2mt can modulate IFNα-induced apoptosis in H9 cells by blocking the cytochrome *c* release from mitochondria and by inhibiting the caspase 3 cleavage and activity. Therefore, the RNase L/IF2mt interaction mediates RNase L mitochondrial nuclease activity, modulates mitochondrial IFNα-induced apoptosis and partially regulates the IFNα antiproliferative activity.

Discussion

In this report, we show that IF2mt interacts with RNase L. In addition, we provide evidence that IF2mt/RNase L interaction is important for mitochondrial mRNA degradation by RNase L and apoptosis induced by IFNα.

IF2mt interacts with RNase L

Earlier observations showed that RNase L activity is regulated by its interaction with other proteins like RLI and eRF3/GSPT1.[21,23,30] To discover new pathways of RNase L regulation and new partners, we performed a yeast two-hybrid screening using the human RNase L as bait and

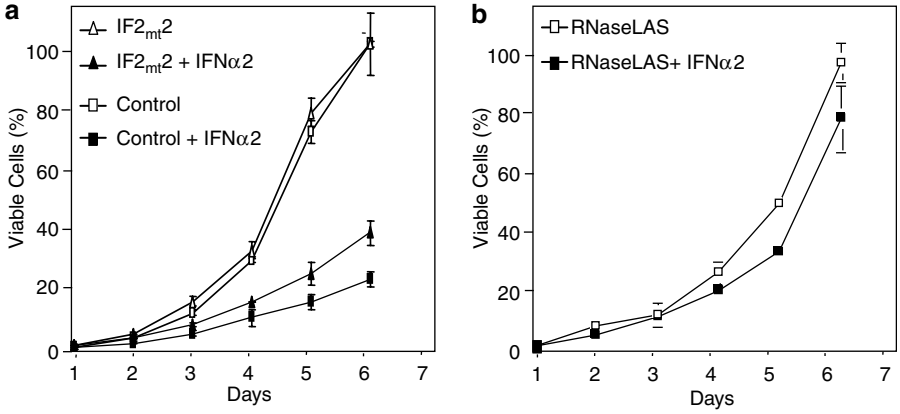

Figure 5 IF2mt overexpression in H9 cells partially reverses IFNα2 growth inhibition. (**a**) H9 cells overexpressing IF2mt (IF2mt2) were untreated (open triangle) or treated with IFNα2 (closed triangle) for 6 days. Control H9 cells were untreated (open square) or treated with IFNα2 (closed square) for 6 days. (**b**) H9 cells overexpressing RNase L antisense (RNaseLAS) were untreated (open square) or treated with IFNα2 (closed square) during 6 days. The cells (in a and b) were counted every day, during 6 days. The experiments were done in triplicate and the standard deviations are indicated on the plots. A value of 100% corresponds to the quantity of viable cells in control cells at day 6

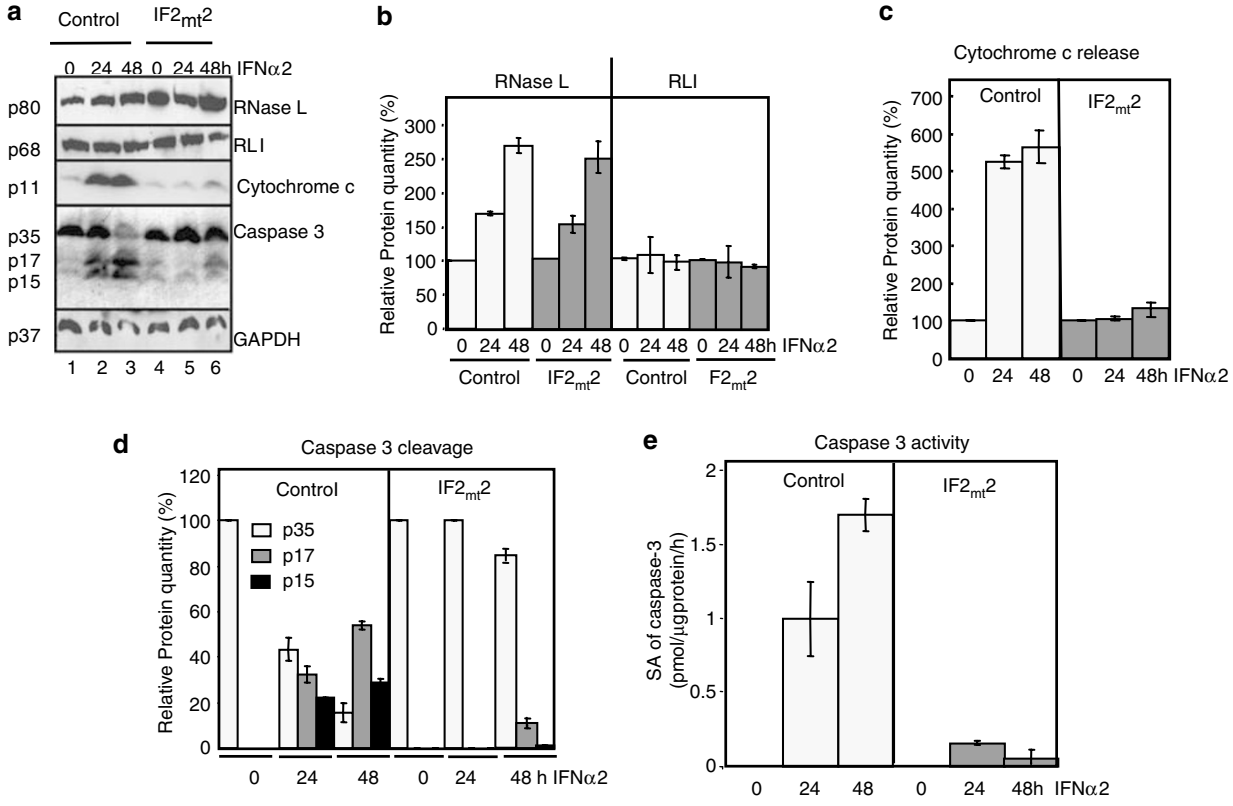

Figure 6 IF2mt overexpression in H9 cells blocks IFNα2-induced apoptosis. (**a**) Western blot analysis for RNase L, RLI, cytochrome *c*, and caspase 3 (pro-caspase 3: p35 and cleaved caspase 3: p17 and p15), and GAPDH on cells extract (200 μg) from H9 control cells or IF2mt2 clone cells treated or not by IFNα2 up to 48 h. Quantification from the Western blot of RNase L and RLI proteins (**b**), cytochrome *c* release in the cytoplasm (**c**) and p35 pro-caspase 3 (white), p17 (grey) and p15 (black) cleaved caspase 3 in control or IF2mt2 clone cells treated or not with IFNα2 up to 48 h (**d**). For (**b**) and (**c**), the value of 100% corresponds to the protein quantity in untreated control cells. For (**d**), the value of 100% corresponds to the total amount of proteins (pro-caspase 3 and cleaved caspase 3) per lane. (**e**) Analysis of caspase 3 specific activity (SA) by measuring the cleavage of the colorimetric substrate peptide Ac-DEVD-pNA in IF2mt2 cells treated or not with IFNα2 and in control H9 cells treated or not with IFNα2 up to 48 h. The liberation of the chromophore pNA is measured by absorbance at 405 nm. All the experiments were done in triplicate and the standard deviation are indicated on the plots

identified the IF2mt as a partner. This result was strengthened by the localization of RNase L in mitochondria[12] and was confirmed *in vitro* by pull-down and co-immunoprecipitation (Figure 1). It is interesting to note that IF2mt/RNase L interaction, contrarily to eRF3/RNase L interaction, is not 2-5A dependent (Figure1, our unpublished observations), allowing its detection in yeast two-hybrid experiments.

RNase L mitochondrial mRNA decay is translation dependent. The identification of IF2mt as a partner of RNase L raises the question of the biological importance of this interaction. IF2mt is a mitochondrial translation initiation factor, whereas RNase L regulates the mitochondrial mRNAs stability.[12,18] Thus, one potential role for IF2mt is to help localizing RNase L to its mRNA target during translation initiation. This possibility was supported by the finding that inhibition of mitochondrial translation by chloramphenicol blocks mitochondrial mRNA decay induced by IFNα2 (Figure 2). This report shows that RNase L regulation of mitochondrial mRNAs is dependent on translation.

RNase L/IF2mt interaction regulates RNase L activity in mitochondria. To examine in more depth the hypothesis that RNase L can regulate mitochondrial mRNA decay through its interaction with IF2mt, we extended the study to cells overexpressing IF2mt (Figures 3–6). We hypothesized that overexpression of IF2mt in cells increases the amount of IF2mt factor available in the mitochondria and titrates RNase L from the mitochondrial translation initiation complex. We found that overexpression of IF2mt in H9 cells blocks mitochondrial mRNAs degradation induced by IFNα2 (Figure 4). This complete stabilization of mitochondrial mRNAs was similar to the stabilization level observed by overexpressing the RLI or an RNase L antisense (Figure 4).[12] Interestingly, the inhibition of RNase L nuclease activity in mitochondria (Figure 4) blocks mitochondrial apoptosis induced by IFNα2 (Figure 6). These results highlight for the first time that degradation of mitochondrial mRNA by RNase L plays a role in mitochondria-dependent apoptosis induced by IFNα2. We propose that the degradation of mitochondrial mRNAs by RNase L will lead to inhibition of mitochondrial protein synthesis. The consequence of such an inhibition will be a loss of mitochondrial membrane potential which results in osmotic swelling and cytochrome *c* release[31] accompanied by caspase-3 cleavage. Taken together, our results show that RNase L/IF2mt interaction mediates RNase L mitochondrial nuclease activity, and consequently, the mitochondrial IFNα-induced apoptosis.

The inhibition of mitochondrial RNase L nuclease activity by IF2mt overexpression reverses IFNα-induced apoptosis and only partially IFNα cell-growth inhibition (compare Figures 5a and 6a). On the contrary, when the cytoplasmic and mitochondrial RNase L activities are inhibited by expression of RNase L antisense, IFNα treatment does not affect cell proliferation (compare Figure 5a and 5b).[12] The difference observed could be explained by the existence of nuclear-encoded mRNAs regulated by RNase L involved in growth inhibition induced by IFNα2, like PKR or antizymes mRNAs.[20,23] Moreover, these results suggest that only the IFNα-induced apoptosis is inhibited in IF2mt2 cells and IFNα could go on to exert its cell-growth inhibition, via other mechanisms than mitochondrial apoptosis. IFNα affect cell proliferation by two mechanisms: apoptosis and cell-growth inhibition.[32] The degradation of mitochondrial mRNA by RNase L seems to be an essential mechanism for the mitochondrial IFNα-induced apoptosis, but less important for the mechanism of cell-growth inhibition of IFNα. This indicates the partial interplay between these two mechanisms.

Cells vary in their sensitivity to IFNα treatment, but the reasons of this difference are not completely understood. This difference in cell sensitivity to IFNα treatment could be due to a defect in one or both of these two mechanisms: apoptosis and cell-growth inhibition.

Model for the mechanism of RNase L regulation of mitochondrial mRNA stability. Previous studies indicate that IF2mt plays a key role in mitochondrial translation initiation (Figure 7a).[33] Mitochondrial mRNA binds to the 28S subunit, which is positioned randomly on the RNA. The mitochondrial translational initiation factor 3 (IF3mt) is postulated to alter the position of the mRNA, promoting the positioning of the 5′ start codon into the P-site. Following the correct positioning of the mRNA, IF2mt promotes the binding of fMet-tRNA. Finally, the 39S subunit joins this complex, which leads to the release of the initiation factors and the formation of the 55S initiation complex (Figure 7a). Based on our findings, we propose the following model describing the RNase L degradation of mitochondrial mRNA (Figure 7b). The IF2mt–RNase L interaction brings RNase L into close association with the mitochondrial mRNA before the assembly of the 55S initiation complex and the release of the initiation factors IF2mt and IF3mt. As the IF2mt–RNase L

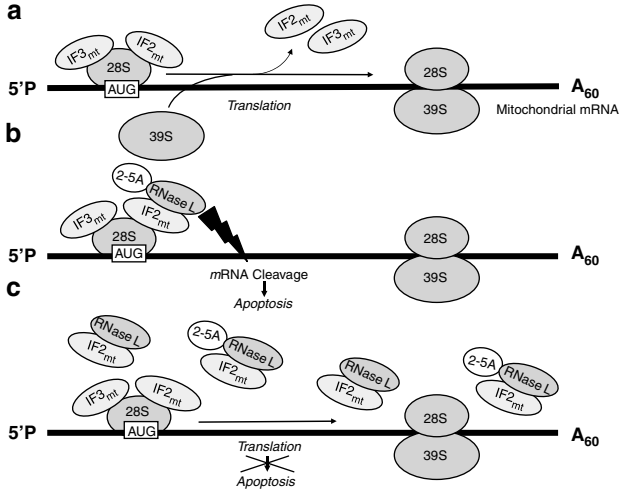

Figure 7 Model for degradation of mitochondrial mRNA by RNase L. (**a**) The mitochondrial mRNA binds to the 28S subunit, but the subunit is positioned randomly on the RNA. The mitochondrial translational initiation factor 3 (IF3mt) is postulated to alter the position of the mRNA, promoting the positioning of the 5 start codon into the P-site. Following the correct positioning of the mRNA, IF2mt promotes the binding of fMet-tRNA. Finally, the 39S subunit joins this complex leading to the release of the initiation factors and the formation of the 55S initiation complex. (**b**) Binding of 2-5A promotes a conformational change in RNase L necessary to induce its nuclease activity. The IF2mt/RNase L interaction brings RNase L into close association with the mitochondrial mRNA where it can act as an endoribonuclease before the assembly of the 55S initiation complex and the release of the initiation factors IF2mt. IFNα treatment induces 2-5A synthesis, which leads to RNase L activation, mitochondrial mRNA degradation and apoptosis mediated by cytochrome *c* release and caspase 3 cleavage. (**c**) An excess level of IF2mt protein traps RNase L away from the mitochondrial translation initiation complex inhibiting mitochondrial mRNA degradation and apoptosis induced by IFNα

interaction does not require 2-5A, RNase L could be recruited to the mitochondrial mRNAs as a latent endoribonuclease. IFNα treatment induces 2-5A synthesis, which leads to RNase L activation, mitochondrial mRNA degradation and apoptosis mediated by cytochrome *c* release and caspase 3 cleavage (Figure 7b). In cells overexpressing IF2mt, our data suggest that an excess level of IF2mt protein traps RNase L away from the mitochondrial translation initiation complex. This causes inhibition of mitochondrial mRNA degradation and apoptosis induced by IFNα (Figure 7c).

Materials and methods

Yeast two-hybrid screening. Yeast two-hybrid screening was carried out using the L40 yeast strain (MATa, trp1, leu2, his3, LYS2::lexA-His3, URA3::lexA-LacZ) harboring *HIS3* and *-gal* reporter genes under the control of upstream LexA DNA-binding site as originally described.[34] The bait consisted of the human *RNase L* open reading frame fused to LexA DNA-binding domain (pBTM116-RNase L). About 350 000 clones of a HeLa cell cDNA library (Clontech, Mountain view, CA, USA) constructed in the GAL4 activation domain vector pGADGH were screened for their interaction with RNase L. The plasmids isolated from positive colonies were amplified on a large scale and the nucleotide sequences were determined using an automat sequencer and analyzed using the BLAST algorithm on EMBL and GenBank database.

Cells. Human H9 lymphocytes (lymphoma, cutoneous T lymphocyte ATCC HTB-176) were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal calf serum (Gibco-BRL). Cells were incubated at 37°C in a 5% CO2, 95% air atmosphere. For cell growth experiments, 10^3 cells/well were seeded in 24-well plates (day 0) and IFNα (500 U/ml) was added on the cells, which must be treated with IFNα after 24 h (day 1). The viable cells were counted on day 1, just before adding IFNα, and each following day during a period of 6 days. Cell growth was determined by counting viable cells as determined by trypan blue exclusion.

Cell extracts. H9 cells were washed twice in ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4 (pH 7.4)), pelleted, resuspended in 2 volumes of hypotonic buffer (0.5% (v/v) Nonidet P-40 (NP-40), 20 mM HEPES (pH 7.5), 10 mM KOAc, 15 mM Mg(OAc)2, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 150 μg/ml leupeptin), incubated 10 min on ice, disrupted with a Dounce homogenizer and centrifuged 10 min at 10 000 $\times$ g. The protein concentration was determined by Bradford reaction.[35]

Expression vectors and transfection. The coding sequences of human IF2mt cDNA[24] were subcloned in pcDNA3neo (Invitrogen, Carlsbad, CA, USA) by standard procedures.[36] Transfected H9 cell clones were isolated by dilution of 1 cell/slot in 96-slot plates in the presence of 1 mg/ml Geneticin (Gibco-BRL). IF2mt has a peptide leader at its NH_2 extremity which is necessary for its mitochondrial localization and its COOH extremity allows its interaction with the ribosome.[25] For these reasons, we stably transfected H9 cells with the coding sequence of untagged IF2mt to avoid a decreased IF2mt activity due to a competition between cellular IF2mt and a less active or inactive IF2mt. IF2mt activity might be critical for the cell, as we could not obtain H9 cells stably transfected with IF2mt antisense construct. Two clones expressing sense IF2mt cDNA were selected and named: IF2mt1 and IF2mt2. An empty pcDNA3 vector-transfected clone was used as a control.

RNA analysis. IF2mt1, IF2mt2 and control cells were plated at 2.0 $\times$ 10^5 cells/ml, treated for 24 or 48 h by human IFNα2 (500 U/ml) or IFNα2 and chloramphenicol (50 μg/ml) and collected for RNA extraction using the guanidine thiocyanate-lithium chloride procedure.[37] Total RNAs (20 μg) were analyzed by Northern blot.[36] After transfer onto nylon membranes (Appligen, Illkirch, Graffenstaden, France), rRNA were revealed with 0.5 M Na acetate (pH 5), 0.04% (w/v) methylene blue. The nylon membranes were then incubated with the appropriate [^{32}P]cDNA probes (as indicated in the figure legends) synthesized using the Multiprime radiolabeling kit (Gibco-BRL) and autoradiographied on a PhospholmagerTM 445-SI (Molecular Dynamics, Sunnyvale, CA, USA).

To determine the stability of mitochondrial mRNAs (ATP6, CYTB, COII), H9 cells were treated with or without IFNα2 (500 U/ml during 24 h) and chloramphenicol (50 μg/ml) and then with actinomycin D (5 μg/ml). Total RNA were isolated at increasing time points after treatment and were analyzed by Northern blot as described above.

Gene expression was detected by RT-PCR using the SuperScript First-strand Synthesis System for RT-PCR (InVitrogen). Briefly, 3 μg of total RNA was denatured at 70°C and then reverse-transcribed by RT enzyme at 42°C for 50 min. Target gene expression was detected by PCR amplification by using following specific primer pairs: sense 5′-CCCGGGGCTGTAGATTCCTT-3′ and antisense 5′-GGGCCTCATTTTCCGGATCT-3′, which hybridize within the 5′ noncoding region of IF2mt, allowing only the amplification of the endogenous IF2mt mRNA; sense 5′-GCATGCCAAAGATGCACAGG-3′ and antisense 5′-TGCTTCTGCCAAAGCCAT CA-3′, which hybridize within the IF2mt coding sequence so that it amplifies the endogenous IF2mt mRNA and the transfected IF2mt mRNA ; sense 5′-GCT GGA AGA CTT AAA CCT GAG GAA GGA-3′ and antisense 5′-AGG CTT CAT TAC ATC GGT CAC AA-3′ for RLI; sense 5′-GGA GAT CCA CAG GAA GTC AAG AGA-3′ and antisense 5′-CAG GAT GGA AGA GAC GAT GAA TG-3′ for RNase L; sense 5′-CAT GTG TGT TGA GAG CTT C-3′ and antisense 5′-GAA AACCAA AGT GGT CAA C-3′ for eF1α; sense 5′-TGG AAG CCT GTC AAA GAG AGA GA-3′ and antisense 5′-TCG ATG AGC TTG ACA TAG ATT TGC-3′ for OAS1; sense 5′-GCT TTG ATG TGC TTC CTG CCT T-3′ and antisense 5′-ACC CCT TTG GCT TCA GTT TCC TT-3′ for OAS2. PCR was performed with 30 cycles in 25 μl of reaction mixture, after 2 min at 94°C cycling conditions were: 30 s at 94°C, 30 s at 58°C, 30 s at 72°C then 7 min at 72°C. PCR products were visualized in 2% agarose gels stained with ethidium bromide. The experiments were done in triplicate, and the standard deviation is indicated on the plots.

Pull-down experiments. Recombinant RNase L tagged with glutathione S-transferase (GST), produced in baculovirus-infected insect cells [38] was incubated 1 h at room temperature with glutathione-sepharose (Amersham Biosciences, Piscataway, NJ, USA) equilibrated in Tris buffer (10 mM Tris-HCl (pH 7.5), 130 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 150 μg/ml leupeptin). After washing three times with Tris buffer supplemented with 0.1% (v/v) NP-40 and 0.2% (w/v) bovine serum albumin, glutathione-sepharose was incubated with rabbit reticulocyte extract (45 μl), for 1 h at room temperature where human IF2mt was translated in presence of ^{35}S-methionine according to manufacturer protocol (Promega, Madison, WI, USA). The mix was centrifuged and the supernatant was considered as the unbound proteins fraction. The glutathione-sepharose was then washed five times with Tris buffer supplemented with 0.1% (v/v) NP-40 and resuspended in 50 μl of gel analysis buffer: 60 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 5% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue, 10% (v/v) glycerol, heated at 95°C during 10 min, pelleted at 10 000 $\times$ g during 2 min. The supernatant was considered as the bound proteins fraction and analyzed by 10% (v/v) SDS-PAGE.[39] Labeled proteins were visualized after autoradiography (PhosphorImager) and proteins bands were quantified by image analysis with the Intelligent Quantifier program (BioImage systems corporation, Jackson, MI, USA).

Co-immunoprecipitation experiments. Human IF2mt was translated in rabbit reticulocyte extract (45 μl) in the presence of ^{35}S-methionine according to manufacturer protocol (Promega). After translation, the reaction was incubated 3 h at room temperature under gentle shaking, with polyclonal antibodies against RNase L (80 μg/ml)[38] or with the irrelevant antibody anti-lexA (1/500°) (a generous gift of Didier Fesquet, CRBM, Montpellier, France) in 450 μl of immunoprecipitation buffer (10 mM Tris-HCL (pH 7.5), 130 mM NaCl, 0.1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 150 μg/ml leupeptin). After addition of protein A-sepharose (Amersham Biosciences), the reaction was incubated overnight at 4°C with gentle shaking. After several washes with the immunoprecipitation buffer, the protein A-sepharose was resuspended in gel analysis buffer, heated at 95°C for 10 min, pelleted at 10 000 $\times$ g for 2 min. The supernatant was analyzed by 10% (v/v) SDS-PAGE and exposure to PhosphorImager.

Western blot analysis. Cytoplasmic extracts (200 μg proteins) were analyzed by 10% (v/v) SDS-PAGE.[39] Proteins were then transferred on a nitrocellulose sheet,[40] blocked in PBS supplemented with 5% (w/v) non-fat milk and then soaked overnight at 4°C with one of the different specific antibodies: rabbit polyclonal antibody against caspase 3 (1/500°) (Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal antibody against RNase L (1 μg/ml) and rabbit polyclonal antibody against RLI (0.4 μg/ml) (Abcam, Cambridge, UK), rabbit polyclonal antibody against cytochrome *c* (0.2 μg/ml) (Santa-Cruz Biotechnology,

Santa Cruz, CA, USA), rabbit polyclonal antibody against GAPDH (40 μg/ml, a generous gift of Guy Cathala, IGMM, Montpellier, France). After several wash with PBST (PBS supplemented with Tween-20 0.05% (v/v)), the nitrocellulose sheet is blocked in non-fat milk and incubated with second antibody linked with horseradish peroxidase (Amersham). The chemiluminescence was enhanced by an ECL kit (SuperSignal West Femto Pierce, Rockford, IL, USA) as described by the manufacturer. Labeled proteins were visualized after autoradiography and proteins bands were quantified by image analysis with the NIH Image program. The experiments were done in triplicate, and the S.D. is indicated on the plots.

Measurement of caspase 3 activity. Caspase 3 activity was measured with the CaspACE Assay System (Promega). Following 24 or 48 h IFNα2 treatment (500 U/ml), cells extracts were prepared as described above. Caspase 3 activity was assayed in 100 μl reaction mixture with 70 μg of cell extracts and a colorimetric caspase 3 substrate Ac-DEVD-pNA (Asp-Glu-Val-Asp-*p*-nitroaniline) according to manufacturer protocol. After incubation of 4 h at 37°C, the liberation of the chromophore pNA was measured at 405 nm. Results are expressed as specific activity of caspase 3 in the cell extract: SA = (pmol pNA liberated per h/μg protein) – (pmol pNA liberated per h/μg protein of control non IFNα-treated cell extract). The experiments were done in triplicate and the standard deviations are indicated on the plots.

Acknowledgements. We are very grateful to D Weil and F Dautry for critical reading of the manuscript. We thank B Lebleu (UMR 5124, Montpellier) for two-hybrid screening facilities at the beginning of this work, G Cathala (UMR 5535, Montpellier) for the gift of polyclonal antibody against GAPDH, G Uzé (UMR 5124, Montpellier) and P Eid (FRE 2937, Villejuif) for the gift of IFNα2. This work was supported by grants from the Association pour la Recherche contre le Cancer (4731) for CB FLR is funded by a postdoctoral fellowship from la Fondation pour la Recherche Medicale.

1. Krammer PH. CD95's deadly mission in the immune system. *Nature* 2000; **407**: 789–795.
2. Twomey C, McCarthy JV. Pathways of apoptosis and importance in development. *J Cell Mol Med* 2005; **9**: 345–359.
3. Meier P, Finch A, Evan G. Apoptosis in development. *Nature* 2000; **407**: 796–801.
4. Kerr IM, Brown RE. pppA2′p5′A2′p5′A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. *Proc Natl Acad Sci USA* 1978; **75**: 256–260.
5. Rebouillat D, Hovanessian AG. The human 2′,5′-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. *J Interferon Cytokine Res* 1999; **19**: 295–308.
6. Floyd SG, Slattery E, Lengyel P. Interferon action: RNA cleavage pattern of a (2′-5′)oligoadenylate — dependent endonuclease. *Science* 1981; **212**: 1030–1032.
7. Zhou A, Hassel BA, Silverman RH. Expression cloning of 2-5A-dependent RNAase: a uniquely regulated mediator of interferon action. *Cell* 1993; **72**: 753–765.
8. Hassel BA, Zhou A, Sotomayor C, Maran A, Silverman RH. A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. *EMBO J* 1993; **12**: 3297–3304.
9. Castelli JC, Hassel BA, Wood KA, Li XL, Amemiya K, Dalakas MC *et al.* A study of the interferon antiviral mechanism: apoptosis activation by the 2-5A system. *J Exp Med* 1997; **186**: 967–972.
10. Zhou A, Paranjape J, Brown TL, Nie H, Naik S, Dong B *et al.* Interferon action and apoptosis are defective in mice devoid of 2′,5′- oligoadenylate-dependent RNase L. *EMBO J* 1997; **16**: 6355–6363.
11. Castelli JC, Hassel BA, Maran A, Paranjape J, Hewitt JA, Li XL *et al.* The role of 2′-5′ oligoadenylate-activated ribonuclease L in apoptosis. *Cell Death Differ* 1998; **5**: 313–320.
12. Le Roy F, Bisbal C, Silhol M, Martinand C, Lebleu B, Salehzada T. The 2-5A/RNase L/RNase L inhibitor (RNI) pathway regulates mitochondrial mRNAs stability in interferon alpha -treated H9 Cells. *J Biol Chem* 2001; **276**: 48473–48482.
13. Diaz GM, Rivas C, Esteban M. Activation of the IFN-inducible enzyme RNase L causes apoptosis of animal cells. *Virology* 1997; **236**: 354–363.
14. Rusch L, Zhou A, Silverman RH. Caspase-dependent apoptosis by 2′,5′-oligoadenylate activation of RNase L is enhanced by IFN-beta. *J Interferon Cytokine Res* 2000; **20**: 1091–1100.
15. Domingo-Gil E, Esteban M. Role of mitochondria in apoptosis induced by the 2-5A system and mechanisms involved. *Apoptosis* 2006; **11**: 725–738.
16. Silverman RH, Skehel JJ, James TC, Wreschner DH, Kerr IM. rRNA cleavage as an index of ppp(A2′p)nA activity in interferon-treated encephalomyocarditis virus-infected cells. *J Virol* 1983; **46**: 1051–1055.
17. Li XL, Blackford JA, Judge CS, Liu M, Xiao W, Kalvakolanu DV *et al.* RNase-L-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response. *J Biol Chem* 2000; **275**: 8880–8888.
18. Chandrasekaran K, Mehrabian Z, Li XL, Hassel B. RNase-L regulates the stability of mitochondrial DNA-encoded mRNAs in mouse embryo fibroblasts. *Biochem Biophys Res Commun* 2004; **325**: 18–23.
19. Bisbal C, Silhol M, Laubenthal H, Kaluza T, Carnac G, Milligan L *et al.* The 2′-5′ oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol Cell Biol* 2000; **20**: 4959–4969.
20. Khabar KS, Siddiqui YM, al-Zoghaibi F, al-Haj L, Dhalla M, Zhou A *et al.* RNase L mediates transient control of the interferon response through modulation of the double-stranded RNA-dependent protein kinase PKR. *J Biol Chem* 2003; **278**: 20124–20132.
21. Bisbal C, Martinand C, Silhol M, Lebleu B, Salehzada T. Cloning and characterization of a RNAse L inhibitor. A new component of the interferon-regulated 2-5A pathway. *J Biol Chem* 1995; **270**: 13308–13317.
22. Shetzline SE, Martinand-Mari C, Reichenbach NL, Buletic Z, Lebleu B, Pfleiderer W *et al.* Structural and functional features of the 37-kDa 2-5A-dependent RNase L in chronic fatigue syndrome. *J Interferon Cytokine Res* 2002; **22**: 443–456.
23. Le Roy F, Salehzada T, Bisbal C, Dougherty JP, Peltz SW. A newly discovered function for RNase L in regulating translation termination. *Nat Struct Mol Biol* 2005; **12**: 505–512.
24. Ma L, Spremulli LL. Cloning and sequence analysis of the human mitochondrial translational initiation factor 2 cDNA. *J Biol Chem* 1995; **270**: 1859–1865.
25. Ma J, Spremulli LL. Expression, purification, and mechanistic studies of bovine mitochondrial translational initiation factor 2. *J Biol Chem* 1996; **271**: 5805–5811.
26. Kim H, You S, Kim IJ, Farris J, Foster LK, Foster DN. Increased mitochondrial-encoded gene transcription in immortal DF-1 cells. *Exp Cell Res* 2001; **265**: 339–347.
27. Kusari J, Tiwari RK, Kumar R, Sen GC. Expression of interferon-inducible genes in RD-114 cells. *J Virol* 1987; **61**: 1524–1531.
28. Yanase N, Ohshima K, Ikegami H, Mizuguchi J. Cytochrome *c* release, mitochondrial membrane depolarization, caspase-3 activation, and Bax-alpha cleavage during IFN-alpha-induced apoptosis in Daudi B lymphoma cells. *J Interferon Cytokine Res* 2000; **20**: 1121–1129.
29. Thyrell L, Erickson S, Zhivotovsky B, Pokrovskaja K, Sangfelt O, Castro J *et al.* Mechanisms of Interferon-alpha induced apoptosis in malignant cells. *Oncogene* 2002; **21**: 1251–1262.
30. Le Roy F, Laskowska A, Silhol M, Salehzada T, Bisbal C. Characterization of RNABP, an RNA binding protein that associates with RNase L. *J Interferon Cytokine Res* 2000; **20**: 635–644.
31. Bernardi P, Scorrano L, Colonna R, Petronilli V, Di LF. Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur J Biochem* 1999; **264**: 687–701.
32. Sangfelt O, Erickson S, Castro J, Heiden T, Einhorn S, Grander D. Induction of apoptosis and inhibition of cell growth are independent responses to interferon-alpha in hematopoietic cell lines. *Cell Growth Differ* 1997; **8**: 343–352.
33. Bhargava K, Spremulli LL. Role of the N- and C-terminal extensions on the activity of mammalian mitochondrial translational initiation factor 3. *Nucleic Acids Res* 2005; **33**: 7011–7018.
34. Chien CT, Bartel PL, Sternglanz R, Fields S. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci USA* 1991; **88**: 9578–9582.
35. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–251.
36. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1982.
37. Cathala G, Savouret JF, Mendez B, West BL, Karin M, Martial JA *et al.* A method for isolation of intact, translationally active ribonucleic acid. *DNA* 1983; **2**: 329–335.
38. Martinand C, Salehzada T, Silhol M, Lebleu B, Bisbal C. RNase L inhibitor (RLI) antisense constructions block partially the down regulation of the 2-5A/RNase L pathway in encephalomyocarditis-virus-(EMCV)-infected cells. *Eur J Biochem* 1998; **254**: 238–247.
39. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
40. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4354.