RNase L Mediates Transient Control of The Interferon Response Through Modulation of The Double-stranded RNA-Dependent Protein Kinase PKR

Khalid S.A. Khabar  
*King Faisal Specialist Hospital and Research Center*

Yunus M. Siddiqui  
*King Faisal Specialist Hospital and Research Center*

Fahad Al-Zoghaibi  
*King Faisal Specialist Hospital and Research Center*

Latifa al-Haj  
*King Faisal Specialist Hospital and Research Center*

Mohammed Dhalla  
*King Faisal Specialist Hospital and Research Center*

Follow this and additional works at: https://engagedscholarship.csuohio.edu/scichem_facpub

Recommended Citation
Khabar, Khalid S.A.; Siddiqui, Yunus M.; Al-Zoghaibi, Fahad; al-Haj, Latifa; Dhalla, Mohammed; Zhou, Aimin; Dong, Beihua; Whitmore, Mark; Paranjape, Jayashree; Al-Ahdal, Mohammed N.; Al-Mohanna, Futwan; Williams, Bryan R.G.; and Silverman, Robert H., "RNase L Mediates Transient Control of The Interferon Response Through Modulation of The Double-stranded RNA-Dependent Protein Kinase PKR" (2003). *Chemistry Faculty Publications*. 405.  
https://engagedscholarship.csuohio.edu/scichem_facpub/405

How does access to this work benefit you? Let us know!
Authors
Khalid S.A. Khabar, Yunus M. Siddiqui, Fahad Al-Zoghaibi, Latifa al-Haj, Mohammed Dhalla, Aimin Zhou, Beihua Dong, Mark Whitmore, Jayashree Paranjape, Mohammed N. Al-Ahdal, Futwan Al-Mohanna, Bryan R.G. Williams, and Robert H. Silverman

This article is available at EngagedScholarship@CSU: https://engagedscholarship.csuohio.edu/scichem_facpub/405
The transient control of diverse biological responses that occurs in response to varied forms of stress is often a highly regulated process. During the interferon (IFN) response, translational repression due to phosphorylation of eukaryotic initiation factor 2α, eIF2α, by the double-stranded RNA-dependent protein kinase, PKR, constitutes a means of inhibiting viral replication. Here we show that the transient nature of the IFN response against acute viral infections is regulated, at least in part, by RNase L. During the IFN antiviral response in RNase L-null cells, PKR mRNA stability was enhanced, PKR induction was increased, and the phosphorylated form of eIF2α appeared with extended kinetics compared with similarly treated wild type cells. An enhanced IFN response in RNase L-null cells was also demonstrated by monitoring inhibition of viral protein synthesis. Furthermore, ectopic expression of RNase L from a plasmid vector prevented the IFN induction of PKR. These results suggest a role for RNase L in the transient control of the IFN response and possibly of other cytokine and stress responses.

Negative feedback mechanisms are essential for maintaining transient responses such as those of the immune response by attenuating undesirable outcomes resulting from prolonged responses. Different negative regulatory pathways of interferon (IFN) and cytokine responses exist in mammalian cells such as those of the suppressors of signals (1) and mRNA turnover regulation by the AU-rich elements in the 3′-untranslated regions (2). The IFN antiviral response is a highly regulated process involving the transient inhibition of viral and cellular protein synthesis. The translational inhibition is mediated predominantly by the activity of PKR, the double-stranded RNA-dependent protein kinase. PKR is a serine/threonine protein kinase of 65 and 68 kDa in murine and human cells, respectively, that is induced by IFN treatment of cells and phosphorylates itself and other proteins, notably eIF2α, in response to viral double-stranded RNA (3). dsRNA is produced as the replicative intermediates of many RNA viruses and also by annealing of complementary RNA strands transcribed from some DNA viruses (3). Phosphorylated eIF2α sequesters the guanine nucleotide exchange factor, eIF2B, which becomes trapped as inactive complex with GDP resulting in translational arrest (4, 5).

Among the principal effectors of the IFN-induced antiviral state are the 2′,5′-oligoadenylate (2-5A) synthetases that convert ATP to 2-5A, activators of RNase L, in response to viral double-stranded RNA (6, 7). Thus, 2-5A is an alarmone that alerts the cells to the presence of virus by signaling to RNase L. Both RNase L and PKR have been implicated in the action of IFN-α against a variety of viruses (reviewed in Refs. 8 and 9). RNase L is widely distributed in different tissues, and it has been suggested that low levels of 2-5A lead to RNase L-mediated selective degradation of viral mRNA (10), whereas higher levels may lead to broader effects such as cleavage of 18 S and 28 S ribosomal RNAs (11). During the course of experiments on the role of RNase L in the inhibition of viral protein synthesis during acute infections, we observed that an absence of RNase L led to selective stabilization of PKR mRNA, extended kinetics of eIF2α phosphorylation, and potent inhibition of viral protein synthesis. Our findings suggest that RNase L truncates and limits the induction of PKR, possibly contributing to the transient nature of the IFN response against viral infections.

EXPERIMENTAL PROCEDURES

Cell Culture, Viral Infections, and IFN Treatments—RNase L–/– and RNase L+/– mouse embryonic fibroblast (MEF) cell lines were of mixed or C57BL/6 genetic backgrounds. The cell lines are post-crisis derivatives of primary MEFs as described previously (12). MEFs were cultured in DMEM with high glucose supplemented with 10% FBS and antibiotics (Invitrogen, Gaithersburg, MD). Bone marrow macrophages collected from the femurs of RNase L–/– and RNase L+/– mice, both on a background of C57BL/6, were cultured in L-cell-conditioned medium for 8 days and plated at a density of 10⁶ cells per 10-cm plate. WISH cell line (HeLa markers) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 supplemented with 10% FBS and antibiotics.

This paper is available on line at http://www.jbc.org
Encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV, Indiana strain) were obtained from the ATCC (Manassas, VA). Viruses were prepared as described previously (20) through 0.22-μm membranes for sterility, and titrated on VERO (African Green Monkey Kidney cell line, ATCC) with resultant titers of 2 × 10^7 and 8 × 10^6 plaque forming units/ml for EMCV and VSV, respectively. Viruses were aliquoted and stored at −70 °C until use.

Cells, primarily over-seeded confluent monolayers of VERO cells, were infected for 1 h at room temperature. After the incubation period, cells were washed, cells were lysed in buffer containing 1% Triton X-100. Lysates were visualized by confocal microscopy using specific antibody to PKR (Santa Cruz Biotechnology Co., Santa Cruz, CA) or by Western blotting. Cells were incubated onto capsule cups for use with a Cytosensor (Molecular Devices, Sunnyvale, CA) overnight at 37 °C in CO₂ incubator. The cells were then transferred into the silicon-containing sensor chambers of the Cytosensor and pumped with running buffer comprising low buffered (1 mM phosphate) RPMI medium supplemented with 1% BSA at a flow rate of 100 μl/min and pump cycle was 2 min. The pump cycle consisted of on and off cycles: in the off cycle the fluid flow was periodically halted allowing a build-up of protons (due to acid metabolites as a result of receptor activation) in the chamber; in the on cycle the pump allowed fluid to be resumed and the acid was flushed out of the chamber. This cycle was repeated, and the basal rate of acidification was first measured in the absence of IFN-α. The acidification rate is then the output of protons from the cells. The acidification rates due to IFN-α were measured; using a 2-min pump protocol and five repeat cycles, before and after IFN-α treatment to determine the relative changes in acidification rates due to IFN-α. The operation of the apparatus was carried out as recommended by the manufacturer. Data were processed by software supplied by the same manufacturer, and data were exported as an Excel spreadsheet file to GraphPad Prism software (San Diego, CA) for graphics.

Measurement of STAT-1 Tyrosine Phosphorylation—Cells (10^5) were harvested by scraping with primary antibody to PKR (Santa Cruz Biotechnology Co., Santa Cruz, CA) and washed to remove antibody non-specific binding. After washing, the cells were lysed in buffer containing 1% Triton X-100. Lysates were clarified in ice-cold lysis buffer. Proteins were separated on SDS-10% acrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA). Membranes were blocked with buffer containing 2% BSA, 0.5% Tween-20 and probed with anti-phospho-eIF-2α antisemur (Cell Signaling Technology, Beverly, MA) incubating the membranes overnight at 4 °C with antibody diluted in the blocking buffer. The antibody specifically detects eIF2α only when phosphorylated at serine-51, because the antibody was raised against a peptide corresponding to residues surrounding Ser-51 of human eIF2α. Secondary antibody (anti-rabbit IgG labeled with horseradish peroxidase) was used for immunodetection and visualization (ECL, Amersham Biosciences). The cells were incubated for a further 1.5 h, media were replaced with methionine-free maintenance medium containing 5 μM of actinomycin D (Sigma, St. Louis, MO). The medium was replaced with cysteine/methionine-free medium supplemented with 6 μCi of [35S]cysteine/methionine (Promix, Amersham Biosciences). The cells were incubated for a further 1.5 h, media were replaced with methionine-free medium containing 5 μCi of [35S]methionine (Amersham Biosciences) and incubated for 1 h. Unadsorbed virus was removed, and cells were incubated for 4 h with maintenance medium containing 5 μg/ml of actinomycin D (Sigma, St. Louis, MO).

Infectious Virus Yield Assays—Confluent cells were infected for 1 h, unadsorbed virus was removed by washing monolayers with PBS containing 2% FBS, and infections were continued in MEM with high glucose supplemented with 5% FBS and antibiotics. Cell lysates were obtained by repeated freezing and thawing, and supernatants containing (containing both intracellular and extracellular viral particles) were collected after centrifugation at 300 × g at 4 °C. Infectious virus in the clarified supernatants were quantitated by plaque assays after titration on confluent monolayers of VERO cells grown in 24-well plates with overlay MEM medium containing agar (Sigma). The plates were fixed and stained with crystal violet, and plaques were counted.

Immunoprecipitation and Autophosphorylation of PKR—Confluent cells (4 × 10^5 cells per well) in 12-well tissue culture dishes (Linbro) were infected with or without IFN-α (1000 units/ml) for different periods of time. Cultures were incubated for 24 h in methionine-free minimal essential medium (Invitrogen) supplemented with 2% dialyzed FBS and 10 μCi of [35S]methionine (Amersham Biosciences). After washing, cells were lysed in buffer containing 1% Triton X-100. Lysates were clarified in ice-cold lysis buffer. Bovine serum albumin (BSA) and immunoprecipitation lysis were immobilized on protein-A-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. PKR was eluted by boiling in gel sample buffer and incubated with γ-[32P]ATP and poly(dI-dC)(poly(dI-dC) in a kinase reaction buffer for 30 min.

SDS-PAGE—Equal amounts, as determined by the Bradford method (Bio-Rad, Richmond, CA), of proteins or equal amounts of the immunoprecipitates were ephosphorylated in 12% SDS-PAGE gels. The gels were fixed overnight, washed, dried, and visualized by autoradiography (Kodak XAR film, Kodak, Rochester, NY) at −70 °C. Protein molecular weight markers (14-C-methylated, 14–220 kDa) were used to verify the size of viral proteins. In the case of phosphorylated STAT-1, protein was visualized using the ECL chemiluminesence kit (Amer- sham Biosciences).

Assay of PKR Levels in Mice Treated with IFN-α—Female RNase L+/− mice were infected for 1 h with or without IFN-α (1000 units/ml) (BBDB) in 200 μl of PBS. Organs (spleen and thymus) were removed and stored before being assayed and assayed for either untreated or IFN-α treated, and 72 h. Protein (300 μg) prepared from frozen (−70 °C) spleens and thymuses were separated by SDS-PAGE, transferred to membrane, and probed with rabbit anti-human PKR (cross-reactive with mouse PKR) and goat anti-rabbit IgG-horseradish peroxidase (HRP, Invitrogen, Carlsbad, CA) and visualized (ECL, Amersham Biosciences). The same blots were re-probed with goat anti-β-actin antiserum (HRP).

Temporal Transfection of Human RNase L—Semi-confluent WISH (HeLa markers) line was assayed for transfection efficiency using green fluorescent protein vector (Invitrogen). Semi-confluent WISH (HeLa markers) was transfected with each plcDNA3.1 vector or pcDNA3.1 containing full-length human RNase L cDNA. Transfections were performed in serum-free medium using LipofectAMINE 2000 (Life Technologies, Inc.) for 6 h followed by replacing the medium with serum-supplemented medium. After 18 h incubation, human recombinant IFN-α (500 IU/ml) was added for additional 18 h. PKR levels were visualized by confocal microscopy using specific antibody to PKR (Santa Cruz Biotechnology Co., Santa Cruz, CA) or by Western blotting.
Quantitative Reverse Transcriptase-PCR—RNase L$^{-/-}$ and RNase L$^{+/+}$ MEFs were treated with IFN-α (DDBB), 1000 units/ml for 16 h. Cells were then treated with 5 μg/ml actinomycin D for different periods of time. Cells were harvested, and total RNA was isolated with TRIzol reagent (Invitrogen). Five μg of total RNA was used to prepare cDNA with Superscript II reverse transcriptase (Invitrogen). Relative levels of PKR mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA were determined using quantitative PCR (Taigon assay) in an ABI Prism 7700 thermal cycler. PCR was performed for 40 cycles according to the manufacturer’s protocol. Data analysis was done using Sequence detector version 1.7 software. Results were expressed as fold difference compared with untreated samples. A rodent glyceraldehyde-3-phosphate dehydrogenase detection kit was obtained from PE Biosystems. PKR forward and reverse primers were prepared at Invitrogen. PKR probe dual-labeled with TAMRA and FAM was synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Primers and probes were selected using the Primer Express 1.0 software from ABI, Inc. The PKR forward primer sequence is 5'-GTT AAA GAG CCC GCC GAA CAT CCT CTA GCG TTG TC-3', and the reverse primer sequence is 5'-CTG CCG GAA CTA-3', whereas the Taqman PKR probe is 5'-CTG CCG GAA CAT CCT CTA GCG TTG TC-3'.

Statistics, Image Analysis, and mRNA Decay Analysis—For comparison between two groups (columns on figures) the Student's paired t test was used. For comparisons among three groups of data (columns on figures), one-way analysis of variance (ANOVA) was used. Whereas, in evaluating two groups of data in consideration of other factors (e.g. in response to different doses of IFN), two-way ANOVA was used. Repeated-measures ANOVA was used for paired experiments. A paired Student's t test for comparison between two sets of paired data was performed. Two-tailed probabilities were reported. Densitometry, band detection, background subtraction, and normalization of images was performed using ImageMaster Software (Amersham Biosciences). The one-phase exponential decay curve analysis (GraphPad Prism) was used to assess mRNA decay kinetics. The equation, y = $y_0 e^{-kt}$ $+$ PLATEAU, describes the kinetics of mRNA decay, x is time, and y may be a concentration, binding, or response. $y_0$ begins equal to $y_0$ $+$ PLATEAU and decreases to PLATEAU with a rate constant K. The half-life of the decay is $t_{1/2}$ = 0.6932/K. $y_0$ and PLATEAU are expressed in the same units as the y axis. K is expressed in the inverse of the units used by the x axis.

RESULTS

PKR Activity in the Presence and Absence of RNase L—During the course of experiments aimed at studying the role of RNase L in controlling protein synthesis during acute viral infections, regulation of PKR was monitored in RNase L$^{-/-}$ and RNase L$^{+/+}$ mouse embryonic fibroblast (MEF) cell lines by different methods. Initially, PKR assays were performed with poly(I)poly(C) as activator and endogenous eIF2α as substrate in extracts of cells treated with IFN-α (1000 units/ml) for different periods of time. Phosphorylation of the α subunit of eIF2α was determined with a specific antibody. In wild type cells, PKR induction showed transient kinetics following IFN treatment, increasing 4-fold by 6 h and returning to baseline levels by 12 h (Fig. 1A). In contrast, in the RNase L$^{-/-}$ cells, PKR appeared with extended kinetics, remaining elevated at 36 h post-IFN addition. There were no changes in levels of total eIF2α in either cell type during IFN treatment (Fig. 1A, middle panel).

To confirm these findings, endogenous levels of eIF2α phosphorylation were determined in intact cells following EMCV infections. The levels of phosphorylated eIF2α were transiently increased in RNase L$^{+/+}$ cells following IFN treatment and infection with EMCV (Fig. 1B). In contrast, levels of phosphorylated eIF2α in infected RNase L-null cells peaked about 2-fold higher when compared with wild type cells (Fig. 1). In addition, after EMCV infections, phosphorylated eIF2α levels decreased rapidly in wild type cells while remaining elevated in RNase L$^{-/-}$ cells (Fig. 1, A and B).

To rule out indirect effects on eIF2α phosphorylation levels possibly mediated by phoshatases, we immunopurified PKR and measured its kinase activity using [$γ$-32P]ATP. There was significantly increased PKR activity as determined by auto-

phosphorylation in extracts of IFN-treated RNase L-null cells when compared with similarly treated wild type cells (Fig. 1C). Therefore, PKR activity as measured by these three approaches was found to be IFN induced to higher levels and for longer periods of time in the absence of RNase L.

Biological Consequences of RNase L Regulation of PKR on Viral Protein Synthesis—We chose encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV), RNA viruses that are sensitive to IFN-α, to determine the biological consequences of RNase L regulation of PKR (16, 17). Profiles of viral and cellular protein synthesis were determined by pulse labeling 1.5 h with [35S]cysteine/methionine in the presence of actinomycin D to suppress host but not viral transcription (14, 15). IFN treatment inhibited viral protein while restoring synthesis of cellular proteins due to suppression of virus-induced shut-off of host cell protein synthesis (14, 15, 18, 19). At high multiplicities of infection (m.o.i. = 10 or 100), the viruses effectively shut-off cellular protein synthesis (Fig. 2, A–C; compare lanes 2 to 1 and lanes 5 to 4). As a result of IFN treatment, there was partial anti-viral activity in wild type cells but not
Virus yields were measured as described under "Experimental Procedures" and represented as a percentage of control (untreated cells, 100%). Data are the average of three independent experiments ± S.E. ***, statistical significance at p < 0.001, using Student’s paired t test. Viruses are EMCV at m.o.i. of 100 (Fig. 2, A–C, lane 3). IFN-α alone had no significant effect on cellular protein synthesis in either cell type (data not shown). Remarkably, the inhibition of EMCV protein synthesis (m.o.i. of 10) was more potent in RNase L−/− MEFs and RNase L−/− MEFs (black columns) and RNase L−/− MEFs (hatched columns) from experiments. Bars denote S.E. ***, statistical significance at p < 0.001, using Student’s paired t test when comparing RNase L−/− to RNase L−/− cell results. 

![Fig. 2. Inhibition of viral protein biosynthesis.](image)

**Fig. 2.** Inhibition of viral protein biosynthesis. RNase L−/− MEFs and RNase L−/− MEFs were treated with mock (cell control; CC), virus (V), or IFN (1000 units/ml) for 20 h followed by virus (IFN+V). Viruses are EMCV at m.o.i. of 10 (A), EMCV at m.o.i. of 100 (B), and VSV at m.o.i. of 10 (C). Protein synthesis in the presence of actinomycin D, which inhibits cellular but not viral RNA synthesis, was monitored as described under “Experimental Procedures”; equal amounts of lysates were loaded onto SDS-PAGE. MW, molecular mass standards (220, 97.4, 66, 46, 30, 21.5, and 14.3 kDa). Representative autoradiograms from three experiments are shown. Arrows indicate positions of viral proteins. RNase L−/− MEFs (black columns) and RNase L−/− MEFs (hatched columns) were first treated with IFN-α (1000 units/ml, 20 h) except control and infected with EMCV (D) or VSV (E). Infectious virus yields were measured as described under “Experimental Procedures.” Data are the average ± S.E. of three independent experiments and represented as percentage of control (untreated cells, 100%). F, virus yield in absence of IFN in RNase L−/− MEFs (black columns) and RNase L−/− MEFs (hatched columns) from three experiments. Bars denote S.E. ***, statistical significance at p < 0.001, using Student’s paired t test when comparing RNase L−/− to RNase L−/− cell results.

**Fig. 3.** PKR levels in RNase L-null and wild type cells. A, MEFs were treated with IFN-α (1000 units/ml) for the indicated periods of time, except for control (0 h), in media containing [35S]methionine. PKR was immunoprecipitated from cell lysates and visualized in autoradiograms. B, confocal microscopy images of PKR induction. Semi-confluent MEF lines were treated with 1000 units/ml IFN-α for 18 h, followed by fixation and staining with primary antibodies to PKR. The fluorescein isothiocyanate-labeled secondary antibodies were used to reveal the primary antibody against PKR and thus indirectly visualize the PKR protein. C, PKR levels were determined in immunoblots from control or IFN-α-treated (2000 units/ml for 24 h) bone marrow macrophages of RNase L−/− (C57BL/6) and RNase L−/− mice of the same genetic background. The lower panel represents graphical representation of data from the upper panel. PKR levels are shown as a percentage of the relative levels of tubulin. D, organs (spleen and thymus) were excised from RNase L−/− and RNase L−/− mice after intraperitoneal injection of 105 units/ml IFN-α for the indicated periods of time. Western blots were performed using antibodies to PKR and β-actin.

PKR Levels in Different Cell Types in Culture and in Mice—To determine whether the extended kinetics of phosphorylated eIF2α in IFN-treated RNase L−/− were due to PKR, levels of PKR protein were determined (Fig. 3A). IFN induction of PKR was increased with extended kinetics (up to 48 h) in the RNase L-null cells compared with IFN-treated wild type cells (Fig. 3A). The PKR protein levels peaked much later than PKR activity (Fig. 1A) suggesting an additional level of regulation.
PKR up-regulation was shown to be responsible for the overexpression of PKR mRNA, which was about 2-fold in primary macrophages compared with wild type macrophages. Western blots of thymus and spleen proteins were then probed with antibody to PKR. In spleens of the RNase L−/− mice, PKR levels remained elevated (by 1.4-fold) after 24 h of IFN treatment, in contrast to the RNase L+/+ mice in which PKR levels returned nearly to baseline at this time point. In thymus glands, PKR reached 1.7-fold higher levels by 16 h of IFN treatment in the RNase L−/− mice compared with IFN treated wild type mice. These experiments confirm extended PKR levels due to absence of RNase L in primary cells of different types.

**Transgenic Transfections of RNase L cDNA Inhibit PKR Induction in Human Cells**—To overexpress RNase L, we used the human cell line, WISH (HeLa markers), which achieved high transfection efficiency (~85%) as observed with green fluorescent protein vector (data not shown). In contrast, the RNase L-null mouse fibroblasts show low transfection efficiency. The transient expression of human RNase L cDNA in WISH cells caused abrogation of IFN-induced PKR protein as assessed with confocal microscopy using specific antibody to PKR (Fig. 4A). Western blots showed that overexpression of RNase L caused reductions of constitutive (40% β-actin-normalized reduction) and IFN-induced PKR protein (70% β-actin-normalized reduction) (Fig. 4B and C). Generally, staining cells with fluorescence-labeled antibodies (confocal microscopy, Fig. 4A) gave lower background than Western blotting (Fig. 4B), because the latter involved denaturation in SDS-PAGE. The RNase L-induced effect on PKR protein levels correlated with PKR mRNA levels (Fig. 4, D and E), there was about 60% β-actin-normalized reduction in IFN-induced PKR mRNA levels (Fig. 4, D and E).

**RNase L Does Not Regulate PKR Expression and Kinetics by Influencing IFN Signaling**—To elucidate the mechanism of PKR overexpression and/or prolongation in RNase L−/− cells, we determined PKR mRNA levels and stability of RNase L−/− and RNase L+/+ cells as a function of time of IFN treatment. Indeed, in RNase L−/− MEFs there was enhanced and prolonged expression of PKR mRNA in RNase L−/− cells in contrast to the transient and much lower levels observed in wild type cells (Fig. 5, A and B). The data showed that PKR mRNA was induced to about 3-fold higher levels in the RNase L−/− cells and remained elevated after 24 h. In contrast, PKR mRNA levels returned to baseline amounts by 24 h in wild type cells (Fig. 5, A and B). Levels of β-actin mRNA and rRNA, monitored for comparison, were unaffected by the absence of RNase L (Fig. 5, A and C). To determine if enhanced IFN signaling was responsible for the overexpression of PKR mRNA, we monitored tyrosine phosphorylation of STAT-1 and receptor-mediated acidification rates (Fig. 5, D and E). In both cell types, there was STAT-1 phosphorylation due to IFN treatment, but no significant differences in the levels of phosphorylated STAT-1 exist between wild type and RNase L−/− cells (Fig. 5D). Additionally, we used the Cytosensor microphysiometer system (CMS), which is a biosensor capable of measuring minute changes in extracellular acidification due to the receptor activation in living cells (21). Both types of cells were placed in the CMS in low buffered running medium, and the acidification rates were monitored until stable baseline rates were obtained. After equilibrium, cells were exposed to IFN-α for 2 min. The acidification rates due to IFN-α treatment were monitored until returning to baseline (Fig. 5E). We were able to measure activation of the cells owing to IFN-α addition in real-time and found that peak acidification occurred in only 3.3 min. The responses to either type of cells are transient, i.e. rapidly return to the baseline (Fig. 5E). We noted that there were no detectable differences in the peaks, acidification rates, or decay of the IFN response between the wild type and RNase L−/− MEFs.

**PKR mRNA Stability Is Affected by the Presence or Absence of RNase L**—To determine if the effect of RNase L on PKR mRNA levels was at the level of mRNA turnover, we blocked transcrip-
tion with actinomycin D. There was an initial increase in PKR mRNA levels in both types of cells when assessed at 1.5 h probably due to the effect of inhibiting synthesis of labile trans-acting proteins (Fig. 6). Similar effects are observed for some AU-rich mRNAs (22, 23). The initial increase in PKR mRNA amounts was consistently observed (n = 4) and thus was not due to cell culture or experimental variations. We performed one-phase exponential decay kinetics analysis 1.5 h after addition of actinomycin D. PKR mRNA decay rates and relative mRNA half-life changes in the two cell types are shown. The vertical arrows indicate the addition of IFN-α.

Both on a genetic background of C57BL/6, to rule out possible nonspecific cell line differences to assess PKR mRNA stability. A real-time PCR assay was used to measure the half-life of the PKR mRNA (Fig. 6C). Relative results were similar to that obtained by Northern blot assays. Specifically, the half-life of PKR mRNA was 4.6 h in Rnase L−/− cells compared with 0.4 h in wild-type cells.

**DISCUSSION**

Our findings demonstrate that the 2-5A/RNase L system negatively regulates PKR expression. A deficiency in RNase L produces enhanced and prolonged IFN induction of PKR due to increased half-life of PKR mRNA. The PKR mRNA stabiliza-
tion appears to be selective and not due to global mRNA stabilization. Furthermore, in IFN-α-treated EMCV-infected RNase L−/− cells, elevated levels of PKR correlate with enhanced eIF2α phosphorylation and superior inhibition of virus replication. We consistently observed enhanced and/or prolonged IFN induction of PKR in the absence of RNase L regardless of the cell type, genetic background, and whether experiments were performed in cell culture or in animals. Also, transient expression of human RNase L in human cells caused selective abrogation of both constitutive and IFN-induced PKR expression. Taken together our results suggest that RNase L negatively modulates the IFN antiviral response and that the transient appearance of phosphorylated eIF2α under some conditions (e.g. viral infections at high m.o.i. values) is, at least partly, a result of the negative feedback mechanism imparted by RNase L. *RNase L Regulation and Inhibition of Viral Protein Synthesis*—PKR action mediates translational arrest in host cells through phosphorylation of eIF-2α and has been previously implicated in innate immunity to EMCV (24, 25), VSV (26), and HSV-1 (17). Mice deficient in PKR have somewhat reduced antiviral responses to IFNs (26, 27), and notably PKR-deficient cells are prone to HSV-1 infection (17). Thus, PKR-mediated eIF-2α phosphorylation acts as mechanism in antiviral immunity during the IFN response but has also been implicated in a variety of responses induced by growth factor deprivation, oxidative stress, the proinflammatory cytokine tumor necrosis factor-α, bacterial lipopolysaccharides, and viral (dsRNA) products (28–30). The biological consequence of the extended kinetics of PKR mRNA expression in the absence of RNase L is reflected in levels of phosphorylated eIF2α. Subsequently, this led to the enhanced inhibition of viral protein synthesis confirming the importance of the PKR/eIF-2α pathway in controlling viruses. Nevertheless, alternative antiviral IFN-stimulated pathways other than PKR might also be affected by the absence of RNase L.

IFN induction of PKR was significantly increased in RNase L−/−cell lines and modestly increased in primary macrophages and organs isolated from the RNase L−/− animals compared with similarly treated wild type cells and mice. Modest increases of PKR in animals as opposed to cells in culture may be due to the in vivo occurrence of low levels of IFN that would reduce the magnitude of induction. It has been suggested that PKR may participate in the autocrine production of IFN (24, 26). This may explain the occasional presence of higher PKR levels in non-IFN-stimulated cells such as those of primary macrophages isolated from RNase L−/− mice. It is unlikely, however, that increased autocrine production of IFN is responsible for the extended translational arrest in IFN-treated RNase L−/− cell line, because the viral protein synthesis assay was performed within 4 h of virus infection in the presence of the transcriptional blocker, actinomycin D. The exogenous expression of human RNase L in human epithelial cell line (WISH, HeLa markers) not only extends the findings of murine cells to human cells, murine and human and murine RNase L share 64% amino acid homology (31), but also offers a direct link between RNase L and PKR pathways.

**RNase L Regulation of PKR Pathway**—Our data show that the RNase L-mediated regulation of PKR pathway is operative
during IFN response (Fig. 2). This may not be the case, however, with overexpression of RNase L, which resulted in significant reduction of both constitutive and IFN-induced PKR. This may be due to the fact that transfected cells release small amounts of IFN. Another requirement for the enhanced inhibition of viral protein synthesis is high multiplicity of infection (m.o.i.). This is concluded from the reconciliation of previous findings (12, 25) with the findings of this paper. Previously, low m.o.i. conditions (e.g. m.o.i. = 0.01) resulted in viral yields that were either nearly identical or modestly reduced in IFN-treated RNase L-null cells compared with wild type cells. In this report, we used high m.o.i. (10 or 100). The RNA-PKR interaction is complex and is dependent on concentration, length, and structure of dsRNA molecules. PKR levels and optimum interactions are necessary for dimerization of PKR for full activity (32–36). Thus, at high m.o.i., the combination of elevated levels of both viral dsRNA intermediates and IFN-induced PKR accounts for enhanced translational arrest leading to superior antiviral action of IFN-α in RNase L-null cells. Indeed, m.o.i. of 100 resulted even in stronger translational arrest than m.o.i. of 10 (Fig. 2, A and B). In acute infections, high m.o.i., such as those used in this study, may be more representative of an ongoing acute infection than experiments with low m.o.i. Very high levels of dsRNA (e.g. > 10 μg/ml) inhibit PKR (35), but these levels do not appear to be obtained in vivo. Conversely, at low m.o.i., antiviral effects of elevated PKR may not be observed due to insufficient dsRNA. Table I summarizes in vivo and cell culture studies on RNase L and PKR gene disruption experiments, whereas Fig. 7 shows an overall scheme of the translational arrest during IFN response to acute virus infection, including the negative regulation of PKR pathway by RNase L reported here.

Regulation of Cellular mRNA by RNase L—The enhanced expression of PKR in the absence of RNase L was related to increased stability of PKR mRNA (Fig. 6). It is unlikely that these effects are due to changes upstream from mRNA expression such as signaling, because there were no notable changes in the functional response of the cells to IFN-α as measured by the microphysiometer system CytoSensor or STAT-1 phosphorylation, the latter event being necessary to the STAT-JAK pathway-mediated transcription of IFN-stimulated genes (37). For RNase L to degrade mRNAs, it needs to be activated by 2-5A (Fig. 7). These results suggest that 2-5A is present in cells treated with IFN but in the absence of viral infections. It is possible that low levels of dsRNA capable of weakly activating 2-5A synthetase are present among cellular RNA (38, 39) leading to generation of low levels of 2-5A. It was previously suggested that RNase L itself, does not cause global degradation of cellular mRNA in intact cells due to low levels of 2-5A, and only 2-5A at rather high concentrations, is needed to degrade cellular mRNA such as ribosomal RNA (10, 11, 40). Indeed, our data show that changes in the PKR mRNA expression are specific when compared with β-actin mRNA and ribosomal RNA expression. Also, differential display analysis showed that there were no general cellular mRNA profile changes as a result of RNase L deficiency. Two recent studies showed that the mRNA stability of ubiquitin-specific protease mRNAs termed ISG43 and ISG15 mRNA were enhanced in RNase L-null cells, but this has not been correlated with IFN action (41). In a separate study (42), it has been concluded that an effect of RNase L on myoblast determination protein 1 mRNA levels was relatively specific, because expression of several other mRNAs was not altered in RNase L inhibitor-transfected HP68 cells. Also, it has been shown that RNase L degrades mitochondrial mRNAs for cytochrome b, ATPase 6, and cytochrome oxidase subunit II, but not glyceraldehyde-3-phosphate dehydrogenase mRNA (which was used as a control) (43). Taken together, the three studies, including ours, provide evidence for a restricted repertoire of RNase L cellular mRNA targets as opposed to global cellular mRNA regulation by RNase L. However, it remains to be determined whether RNase L selectively affects the turnover of specific sets of mRNAs in the cell.

Our findings support a novel role for RNase L in the control of PKR mRNA stability and therefore, in the transient regulation of PKR levels in IFN-treated cells. The control of mRNA stability is one mechanism that ensures the transient nature of processes such as immune response, cellular growth, differentiation, and responses to external stimuli. RNase L-induced transient responses of PKR activation may be a means of avoiding induction of stress pathways (e.g. activation transcription factor) elicited by eIF2α phosphorylation. Results suggest that RNase L may mediate the negative regulation of the IFN response during acute viral infections and perhaps in other cellular stress responses.

Acknowledgments—We thank Maud Dziemiri, Fehmina Chaudhury, and Stacie Saleh for excellent technical assistance. We also thank Dr. Katherine Collison for reviewing the manuscript.

REFERENCES
1. Greenhalgh, C. J., and Hilton, D. J. (2001) J. Leukoc. Biol. 70, 348–356
2. Bakkevet, T., Frevel, M., Williams, B. R. G., Greer, W., and Khabar, K. S. A. (2003) Nucleic Acids Res. 31, 446–454
3. Bakheet, T., Frevel, M., Williams, B. R. G., Greer, W., and Khabar, K. S. A. (2003) Nucleic Acids Res. 31, 446–454
4. Meurs, E., Chong, K., Galabra, J., Thomas, N. S., Kerr, I. M., Williams, B. R., and Hovanessian, A. G. (1990) Cell 62, 379–390
5. Biron, C. A., and Sen, G. (2001) in Fields Virology (Knipe, D. M., and Howley, P. M., eds) 4th Ed., pp. 321–352, Lippincott Williams & Wilkins, Philadelphia
6. Bakheet, T., Frevel, M., Williams, B. R. G., Greer, W., and Khabar, K. S. A. (2003) Nucleic Acids Res. 31, 446–454
7. Wreschner, D. H., James, T. C., Silverman, R. H., and Kerr, I. M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 256–260
8. Panniers, R., and Henshaw, E. C. (1983) Annu. Rev. Biochem. 52, 227–264
9. Thomas, N. S., Kerr, I. M., Williams, B. R., and Hovanessian, A. G. (1992) J. Virol. 66, 5804–5814
10. Chen, C. Y., Xu, N., and Shyu, A. B. (1995) Mol. Cell. Biol. 15, 2771–2782
11. Khabar, K. S. A., Siddiqui, Y., Zhou, A., Al-Ahdal, M. N., Der, S. D., Nakamura, T., Howley, P. M., eds) 4th Ed., pp. 321–352, Lippincott Williams & Wilkins Publishers, Philadelphia
12. Khabar, K. S. A., Siddiqui, Y., Zhou, A., Al-Ahdal, M. N., Der, S. D., Nakamura, T., Howley, P. M., eds) 4th Ed., pp. 321–352, Lippincott Williams & Wilkins Publishers, Philadelphia
30. Der, S. D., Yang, Y. L., Weissmann, C., and Williams, B. R. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 3279–3283
31. Zhou, A., Nie, H., and Silverman, R. H. (2000) Mamm. Genome 11, 989–992
32. Wu, S., and Kaufman, R. J. (1997) J. Biol. Chem. 272, 1291–1296
33. Pe'ery, T., and Mathews, M. B. (1997) Methods 11, 371–381
34. Ehrenfeld, E., and Hunt, T. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1073–1078
35. Sharp, T. V., Xiao, Q., Jeffrey, I., Gewert, D. R., and Clemens, M. J. (1993) Eur. J. Biochem. 214, 945–948
36. Patel, R. C., Stanton, P., McMillan, N. M., Williams, B. R., and Sen, G. C. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 8283–8287
37. Darnel, J. J., Kerr, I., and Stark, G. (1994) Science 264, 1415–1421
38. Hartmann, R., Norby, P. L., Martensen, P. M., Jorgensen, P., James, M. C., Jacobsen, C., Moestrup, S. K., Clemens, M. J., and Justesen, J. (1998) J. Biol. Chem. 273, 3236–3246
39. Pratt, G., Galpines, A., Sharp, N., Palmer, S., and Clemens, M. J. (1988). Nucleic Acids Res. 16, 3497–3510
40. Cirino, N. M., Li, G., Xiao, W., Torrence, P. F., and Silverman, R. H. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 1937–1942
41. Li, X. L., Blackford, J. A., Judge, C. S., Liu, M., Xiao, W., Kalvakolanu, D. V., and Hassel, B. A. (2000) J. Biol. Chem. 275, 8880–8888
42. Bisbal, C., Silhol, M., Laubenthal, H., Kaluza, T., Carnac, G., Milligan, L., Le Roy, F., and Salehzada, T. (2000) Mol. Cell. Biol. 20, 4959–4969
43. Le Roy, F., Bisbal, C., Silhol, M., Martinand, C., Lebleu, B., and Salehzada, T. (2001) J. Biol. Chem. 276, 48473–48482
44. Haghighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T., and Sonenberg, N. (1996) J. Virol. 70, 8444–8450
45. Kleijn, M., Vrins, C. L., Voorma, H. O., and Thomas, A. A. (1996) Virology 217, 486–494
RNase L Mediates Transient Control of the Interferon Response through Modulation of the Double-stranded RNA-dependent Protein Kinase PKR

Khalid S. A. Khabar, Yunus M. Siddiqui, Fahad Al-Zoghaibi, Latifa Al-Haj, Mohammed Dhalla, Aimin Zhou, Beihua Dong, Mark Whitmore, Jayashree Paranjape, Mohammed N. Al-Ahdal, Futwan Al-Mohanna, Bryan R. G. Williams and Robert H. Silverman

J. Biol. Chem. 2003, 278:20124-20132.
doi: 10.1074/jbc.M208766200 originally published online February 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M208766200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 26 of which can be accessed free at
http://www.jbc.org/content/278/22/20124.full.html#ref-list-1