An Apoptotic Signaling Pathway in the Interferon Antiviral Response Mediated by RNase L and c-Jun NH$_2$-terminal Kinase*

Geqiang Li‡§, Ying Xiang§, Kanaga Sabapathy¶, and Robert H. Silverman§

From the §Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, ¶Department of Cancer Biology, NB40, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, 44195, and Laboratory of Molecular Carcinogenesis, Division of Cellular and Molecular Research, 11 Hospital Drive, National Cancer Centre, Singapore 169610.

Cellular stress responses induced during viral infections are critical to the health and survival of organisms. In higher vertebrates, interferons (IFNs) mediate the innate antiviral response in part through the activation of RNase L, a uniquely regulated enzyme. RNase L is activated by 5'-phosphorylated, 2'-5' oligoadenylates (2–5A) produced from IFN-inducible and double stranded RNA-dependent synthetases. We show that viral activation of the c-Jun NH$_2$-terminal kinases (JNK) family of MAP kinases and viral induction of apoptosis are both deficient in mouse cells lacking RNase L. Also, JNK phosphorylation in response to 2–5A was greatly reduced in RNase L$^{-/-}$ mouse cells. In addition, 2–5A treatment of the human ovarian carcinoma cell line, Hey1b, resulted in specific ribosomal RNA cleavage products coinciding with JNK activation. Furthermore, suppression of JNK activity with the chemical inhibitor, SP600125, prevented apoptosis induced by 2–5A. In contrast, inhibition of alternative MAP kinases, p38 and ERK, failed to prevent 2–5A-mediated apoptosis. Short interfering RNA to JNK1/JNK2 mRNAs resulted in JNK ablation while also suppressing 2–5A-mediated apoptosis. Moreover, Jnk$^{-/-}$ cells were highly resistant to the apoptotic effects of IFN and 2–5A. These findings suggest that JNK and RNase L function in an integrated signaling pathway during the IFN response that leads to elimination of virus-infected cells through apoptosis.

The c-Jun NH$_2$-terminal kinase (JNK)$^3$ family of MAP kinases relays signals from a wide range of extracellular stimuli including viruses, cytokines, and environmental stress (1–4). The three JNK genes (Jnk1, Jnk2, and Jnk3) encode ten different molecular mass species of JNK as a result of alternative splicing, including 55- and 46-kDa isoforms from each gene (5). Mice with homologous disruptions of the individual Jnk genes are viable, as are Jnk1$^{-/-}$ Jnk3$^{-/-}$ and Jnk2$^{-/-}$ Jnk3$^{-/-}$ double gene knockout mice (6). In contrast, combined loss of ubiquitously expressed JNK1 and JNK2 results in embryonic lethality associated with altered neuronal apoptosis and exencephaly (6, 7). JNK3 is present mostly in brain, therefore Jnk1$^{-/-}$ Jnk2$^{-/-}$ mouse embryonic fibroblasts (MEFs) lack JNK and are an important resource for determining the biologic functions of JNK (6, 7). For example, Jnk1$^{-/-}$ Jnk2$^{-/-}$ MEFs are deficient in both activator protein-1 transcriptional activity and stress-induced apoptosis (8, 9).

JNKs often play a critical role in controlling the balance between cell survival and death. For instance, JNK1 signals cell survival in transformed B lymphocytes mediated in part by increased Bcl2 expression (10). In contrast, sustained activation of JNK in the absence of cell survival signals results in apoptosis (11). A major function of JNK is regulation of the activator protein-1 transcription factor through phosphorylation of c-Jun and related proteins (reviewed in Ref. 5). However, in addition to controlling new transcription JNKs participate in apoptotic signaling by regulating the activities of pre-existing Bcl2-related signals that mediate mitochondrial release of cytochrome c and subsequent caspase activation (8). For example, proteolytic activation of Bid, a pro-apoptotic BH3-only Bcl2-related protein, occurs in UV-treated wild type cells but not in similarly treated Jnk1$^{-/-}$ Jnk2$^{-/-}$ MEFs. Furthermore, JNK activation failed to induce death of cells deficient in the pro-apoptotic proteins, Bax and Bak (11). The possible role of JNK in regulating apoptosis through Bcl2 and Bcl-x$_L$, phosphorylation is controversial, in part because results vary as to whether apoptosis would be enhanced or suppressed (12–14). In neurons, the BH3-only Bcl2 member, Bim, and JNK are both implicated in apoptosis caused by nerve growth factor deprivation (15, 16). Although these studies suggest that JNK can control certain Bcl2-related molecules at different levels, the molecular mechanism(s) by which JNK directly or indirectly controls activation of different Bcl2 members remain to be elucidated.

Among the types of cellular stress stimuli that activate JNKs are agents that damage cellular RNA, in particular ribosomal RNA (rRNA). For example, ribotoxins (α-sarcin and ricin A chain) and UV that damage the 3’ end of the large (28 S) rRNA activate JNK (17–19). JNK is also activated by double stranded RNA (dsRNA) treatment of cells, which results in the cleavage of 28 S rRNA by RNase L (20). dsRNA, often viral in origin, activates the IFN-inducible 2–5A synthetases that convert ATP to PP$_i$ and a series of 2’-5’ oligoadenylates (ppA(p2)5’A)$_n$, n = 2 $\leq$ 4) collectively referred to as 2–5A (see Ref. 21; reviewed in...
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RNase L Activity in Intact Cells—Hey1b cells were transfected with 5 μM 2–5A using LipofectAMINE 2000. At the indicated times, the total RNase L was isolated from transfected cells using Trizol reagent (Invitrogen) and quantitated by measuring absorbance at 260 nm. RNA (2 μg) was separated on RNA chips and analyzed with Bioanalyzer 2100 (Agilent Technologies). The peak areas of 28S and 18S rRNA and their main cleavage products were determined by using the Bio Sizing (version A.10.30 S1220) program (Agilent Biotechnologies).

RNase L Activity in a Cell-free System—RNase L activity was determined by the fluorescence resonance energy transfer (FRET) method. The assay uses recombinant human RNase L produced in insect cells from a baculovirus vector and purified to homogeneity with three successive fast protein liquid chromatography columns (24). The cleavable substrate consists of a 36-nucleotide synthetic oligoribonucleotide sequence derived from respiratory syncytial virus with the fluorophore, FAM, at the 5′ terminus and black hole quencher-1 (BHQ-1) at the 3′ terminus (synthesized at Integrated DNA Technologies). The RNA sequence contains several cleavage sites for RNase L (UU or UA). The reaction buffer contains 100 mM RNA probe, 25 mM RNase L, 25 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM MgCl2, 50 μM ATP, and 7 mM Mg-acetate with 3% DMSO. The reaction mixture was incubated for 2 h at 37 °C and then analyzed using an ABI 380B automated DNA synthesizer and were purified by high performance liquid chromatography.

Cells and Cell Culture—The human ovarian carcinoma cell line, Hey1b (a gift from A. Marks and Y. Xu), was grown in RPMI 1640 medium with 10% fetal bovine serum. MEFs were grown in Dulbecco’s modified minimal essential medium with 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin, 2 mM l-glutamine, and non-essential amino acids. Immortalized (post-crisis) RNase L−/− cells were described previously (27). In addition, immortalized (post-crisis) RNase L−/−/H9262 and fibroblasts (27). In addition, myocarditis virus (EMCV) and are deficient in apoptosis in −/−Sustained activation of RNase L by 2′−5′A kinase-like and a ribonuclease domain in the C-terminal half of RNase L (2−5A®RNA) susceptibility allele to the RNase L gene (reviewed in Ref. 22). The only well established function of 2′−5′A kinase is essential for apoptosis in response to RNase L activation. Here we demonstrate involvement of RNase L in JNK activation during viral infections, and furthermore we show that JNK is essential for apoptosis in response to RNase L activation.

MATERIALS AND METHODS

Reagents and Antibodies—The JNK inhibitor, SP600125, and p38 inhibitor, SB202190, were from Calbiochem, whereas p38 inhibitor, SB203580, and the ERK1/2 inhibitor, PD98059, were from Bioworld. Human recombinant human IFN-α DDB, active on both human and mouse cells, was a generous gift from H. K. Hochberger, H. K. Novartis Pharma AG (32). The JNK1/2 siRNA (5′-AAAGAAAAGGUUCUACCUCUUUTT-3′ and 5′-AGAGUAGACGACUUCUUUTT-3′) target a common sequence in both Jnk1 and Jnk2 mRNA (377 nucleotides downstream from the start codon) and was synthesized by Xeragon Inc. The phospho-SAPK/JNK (Thr-183/Tyr-185) antibody and antibodies to total JNK, total PARP, cleaved PARP, total p38, phospho-p38, total ERK, and phospho-ERK were from Cell Signaling Technology, Inc. The antibody to β-actin was from Santa Cruz Biotechnology, Inc. or Sigma. Monoclonal antibody to human RNase L was reported by us previously (24). Natural 2–5A (p(A2′p)A, where n = 1 to 3) was prepared enzymatically from ATP using hexahistidine tagged and purified recombinant 42-kDa 2′−5′A synthetase (a gift from R. Hartmann, Cleveland) (33). The “natural 2–5A” is a mixture of ppp(A2′p)A, in the following proportions: n = 1, 42%; n = 2, 41%; n = 3, 10%; n = 4, 2%. Individual 2–5A oligomers were purified using reverse phase high performance liquid chromatography. Phosphorothioate analogs of 2–5A were prepared using an ABI 380B automated DNA synthesizer and were purified by high performance liquid chromatography.

Viral Infections—Cells were grown overnight, and medium was removed and replaced with serum-free medium containing EMCV (Amer- ican Culture Collection, Rockville, MD) at a multiplicity of infection (m.o.i.) of 1 to 2 for 0.5 h at which time cells were washed, and medium with 10% fetal bovine serum was added. Induction of RNase L expression from inducible vectors was performed by pre-incubating cells with 5 μM panoster- one for 16 h prior to EMCV infections.

Measuring Protein Synthesis in Intact Cells—Rates of cellular protein synthesis were determined as described previously (33). Briefly, cells were transfected with p53-A(2′p)A, for 3 h at 37 °C. After removing medium and washing cells with phosphate-buffered saline, RPMI 1640 medium lacking methionine (Speciality Media, Phillipsburg, NJ) and supplemented with 0.3 μCi per ml of [35S]methionine (>1000 Ci/mmol, Amersham Biosciences) was added, and cells were incubated for a further 2 h. Medium was removed, and protein was precipitated with 5% (v/v) trichloroacetic acid as described (33). Precipitated protein was solubilized with 0.5 mM NaOH and neutralized, and radioactivity was determined by liquid scintillation counting.

RNA was isolated from transfected cells using Trizol reagent (Invitro- gen) and quantitated by measuring absorbance at 260 nm. RNA (2 μg) was separated on RNA chips and analyzed with Bioanalyzer 2100 (Agilent Technologies). The peak areas of 28S and 18S rRNA and their main cleavage products were determined by using the Bio Sizing (version A.10.30 S1220) program (Agilent Biotechnologies).

The viability of Hey1b cells were determined by trypan blue exclusion. The cell extracts (200 μg of protein) were incubated with the probe (0.02 μCi/750 Ci/mmol) on ice for 60 min and then under 308 nm of light on ice for 60 min. Protein separation was by electrophoresis on 4–2% acrylamide gels. Quantitation was by phosphorimage analysis.

Cell Viability Assay—The viability of Hey1b cells were determined by using the colorimetric Cell titer 96A QUANT™Cell Proliferation (tetra- zolium conversion) assay (Promega). Hey1b cells were seeded (1.5 × 104 cells per well) into 96-well plates. At various times of treatment with p53-A(2′p)A, the Cell titer 96A QUANT™reagent (Promega) was added to each well, incubated at 37 °C for 2 h, and absorbance was measured at 490 nm with a 96-well plate reader (model Spectra max 340; Molecular Devices).

Western Blots—Cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na3EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyro- phosphate, 1 mM β-glycerophosphate, 1 mM NaF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Insoluble material was removed by centrifugation at 10,000 × g (at 4 °C for 10 min). Protein concentrations in the supernatants were determined by the method of Bradford (34).

6Z. Wang and R. H. Silverman, unpublished data.

6C. Thakur, Z. Xu, and R. H. Silverman, unpublished data.
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RESULTS

Viral Activation of JNK and Apoptosis Are Deficient in Cells Lacking RNase L—To determine the impact of RNase L on viral-induced apoptosis, RNase L+/+ or RNase L−/− MEFs were infected with EMCV at an m.o.i. of 2 and were then monitored for DNA breakage by FACS TUNEL assays at 8 h post-infection. Results show about 34% of wild type cells became TUNEL-positive after infection, in contrast to only 8% of cells lacking RNase L (Fig. 1B). In addition, in RNase L−/− cells induced to express full-length human RNase L (from plasmid pINDRNase L), the level of virus-induced apoptosis increased to about 20% whereas induced expression of a truncated form of RNase L lacking the endoribonuclease domain (from plasmid pINDRNase LΔEN) produced only about 11% TUNEL-positive cells (Fig. 1, A and B). These findings show that RNase L contributes to EMCV-induced apoptosis in MEFs cells. EMCV infections (m.o.i. = 1) of wild type and the reconstituted (pINDRNase L) MEFs also caused robust phosphorylation of JNK (46- and 54-kDa isoforms) by 6 h post-infection correlating with cleavage of the death substrate PARP (Fig. 1C). In contrast, at the same time point post-infection in the RNase L−/− MEFs there was relatively weak JNK phosphorylation (about 4-fold lower than in the RNase L+/+ and pINDRNase L−/− cells as determined by densitometry) and little or no PARP cleavage. A more modest deficiency in virus-induction of JNK phosphorylation was observed in cells expressing the nuclease-deleted polypeptide, RNase LΔEN, and although a low level of PARP cleavage was present even in the absence of virus there was no increase until a later time (8 h), post-infection. Previously we showed using the same cell lines that the RNase L−/− cells contained the same levels of JNK as wild type MEFs (20). Therefore, these results demonstrate that RNase L contributes to JNK phosphorylation correlating with cell death during EMCV infections.
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2-5A Activation of RNase L Results in Stimulation of JNK and Apoptosis—To determine the specific contribution of RNase L to JNK activation and apoptosis, we transfected cells with 2-5A. Transfection of tetrameric 2-5A, p(A2)p5A (10 μM for 4 h), caused JNK phosphorylation in RNase L−/− cells but not RNase L+/− cells (Fig. 2A, compare lanes 2 and 4). Similar

findings were seen in ponasterone-induced pINDRNase L+ cells expressing full-length wild type RNase L but not in the uninduced cells (Fig. 2A, compare lanes 6 and 8) (34). The ribonuclease domain was necessary for 2-5A induction of JNK as there was no JNK phosphorylation in ponasterone-treated RNase L−/− cells containing pINDRNase L−/− (Fig. 2A, lane 12).

To extend these findings to human cells, studies were performed on the ovarian carcinoma cell line, Hey1b, treated with biostable phosphorothioate 2-5A analogs. A control compound, the diadenylate psA2sp5A, which is unable to activate RNase L, failed to induce PARP cleavage or to cause significant JNK phosphorylation (Fig. 2B, lanes 2–6). In contrast, transfection with the active 2-5A analog, ps(A2)ps5A, resulted in phosphorylation of JNK and c-Jun phosphorylation in a time- and concentration-dependent manner (Fig. 2, B and C). Activation of JNK peaked by 3.5 h after 2-5A transfection, preceding the cleavage of PARP by several hours (Fig. 2B, lanes 7–11).

MAP kinase p38, on the other hand, was not phosphorylated in the Hey1b cells treated with 2-5A. In contrast exposing cells to UV light did activate p38 in these cells (Fig. 2D, lane 14). The tetramer, ps(A2)ps5p5A, caused a dose-dependent (up to 8 μM) inhibition of protein synthesis, <30% of the control, whereas the same concentration of the diadenylate, psA2sp5A, only reduced the protein synthesis rate to 88% of that observed in the untreated cells (Fig. 2E).

To confirm the requirement of ribonuclease activity for apoptosis, we transiently transfected HeLa M cells, which contain low endogenous levels of RNase L, with cDNAs for either wild type RNase L or missense mutant RNase LR667A lacking ribonuclease activity (see Fig. 1A and Fig. 3) (38). PARP cleavage, 55%, was observed at 24 h post-treatment with 2-5A of the control cells (Fig. 3, lane 3). In contrast, in HeLa M cells transfected with wild type RNase L there was 68% PARP cleavage at 9 h and nearly complete cleavage by 24 h post-treatment with 2-5A. On the other hand, transfection with the RNase LR667A showed only slight levels of PARP cleavage, 8% at 24 h, possibly a nonspecific response because of protein over-expression (Fig. 3, lanes 8 and 9). These findings suggest that the ribonuclease activity of RNase L contributes to apoptosis.

JNK activation as measured by c-Jun phosphorylation was compared with rRNA cleavage as a function of time (Fig. 4). The inactive, dimeric form of 2-5A (p(A2)p5A) did not induce JNK activation or rRNA cleavages in the Hey1b cells (Fig. 4, lanes 1–5). In contrast, treatment with natural 2-5A resulted in appearance of both specific rRNA cleavage products and c-Jun phosphorylation beginning by 1 to 2 h post 2-5A treatment (Fig. 4, B, lanes 6–11, and C). To determine whether JNK activation by RNase L required ongoing protein synthesis, cells were pretreated with cycloheximide (10 μg per ml) for 30 min.

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prior to transfection with 2–5A (5 μM) for 3 h (Fig. 5). Cycloheximide treatment by itself caused only weak activation of JNK as measured by c-Jun phosphorylation (Fig. 5, lane 3). In contrast, strong phosphorylation of c-Jun phosphorylation occurred in response to 2–5A treatment regardless of prior inhibition of protein synthesis by cycloheximide treatment (Fig. 5, lanes 2 and 4). Indeed, c-Jun phosphorylation was potentiated by cycloheximide treatment. These results indicate that JNK activation by RNase L does not require ongoing protein synthesis and therefore differs from the ribotoxic stress response (17–20).

Inhibition of JNK Impairs RNase L-induced Apoptosis—To determine whether there was a causal relationship between JNK activation and apoptosis, Hey1b cells were pre-treated with an anthrapyrazolone inhibitor. SP600125 is a reversible, ATP-competitive inhibitor for JNKs and other several other kinases (39, 40). Hey1b cells were pre-incubated 1 h in the absence or presence of different amounts of SP600125 prior to treatments with the active 2–5A analog, psA(2′ps5′A)2. JNK activity was measured by the appearance of phosphorylated c-Jun after 3.5 h of 2–5A treatment. At 10 μM SP600125, activation of JNK was inhibited (Fig. 6A, lane 5, upper panel). In addition, cleavage of PARP measured after 12 h was also inhibited by 10 μM SP600125. Accordingly, cell viability as measured by MTS (tetrazolium conversion) assays, was greatly reduced by 9 h of treatment with 2–5A alone, but cell viability remained unaffected when cells were preincubated with SP600125 prior to 2–5A treatments (Fig. 6B). To determine whether the effect SP600125 directly inhibited RNase L, cell-free system assays were performed. Purified RNase L was incubated with 2–5A in the presence or absence of SP600125. Cleavage of a dual labeled RNA substrate was then measured by the FRET method. Results showed that SP600125 had no effect on RNase L activity (Fig. 6C). Although the JNK inhibitor effectively prevented apoptosis, inhibitors of p38 (SB202190) and SB203580) and ERK (PD98059) had no effect on apoptosis induced by 2–5A as measured by PARP cleavage (Fig. 6D). Therefore, 2–5A-mediated apoptosis requires JNK, but not p38 or ERK.

Ablation of JNK Suppresses Apoptosis Induced by 2–5A Activation of RNase L—Because SP600125 is not specific for JNKs (40), it was necessary to confirm involvement of JNKs by other means. To verify the role of JNKs in RNase L-induced apoptosis, JNK1 and JNK2 were simultaneously ablated in Hey1b cells by transfecting with an siRNA oligonucleotide to a Jnk1 or Jnk2 gene. As a control, negative control siRNA was transfected into Hey1b cells (Fig. 7). Down-regulation in the levels of the JNKs prevented c-Jun fusion protein phosphorylation and also prevented PARP cleavage 9 h after 2–5A treatment (Fig. 7). In contrast, as a control for nonspecific effects, siRNA to luciferase mRNA had no effect on 2–5A induction of JNK activation or PARP cleavage (data not shown).

To provide further evidence for involvement of JNKs in RNase L-mediated apoptosis, Jnk1–/– Jnk2–/– double gene knockout MEFs were used. JNK activity measured by in vitro phosphorylation of c-Jun fusion protein was induced by natural 2–5A (10 μM) in wild type MEFs but was absent in the Jnk1–/– Jnk2–/– cells (Fig. 8A). In addition, 8% PARP cleavage was apparent by 6 h of 2–5A treatment increasing to 27 and 82% cleavage by 9 and 24 h in the wild type cells. In contrast, there was no observable PARP cleavage at 6 h and only 32% cleavage after 24 h in the Jnk1–/– Jnk2–/– cells treated with 2–5A (Fig. 8B). Apoptosis in response to 10 μM natural 2–5A for 24 h was 8% in wild type MEFs and only 3% in the Jnk1–/– Jnk2–/– cells as determined by FACS TUNEL assays. IFN treatments prior to 2–5A transfections are known to enhance apoptosis (27). Although IFN-α treatments (1,000 units per ml) alone did not induce apoptosis in either wild type or Jnk1–/– Jnk2–/– cells, treatment with IFN-α prior to 2–5A transfection caused a large increase in apoptosis (to about 38%) in the wild type cells. In contrast, the combination IFN and 2–5A treatments only marginally increased the level of apoptosis in the Jnk1–/– Jnk2–/– cells, to 4.5% (Fig. 8C). The IFN stimulation of 2–5A-mediated apoptosis was because of a large IFN-induction of RNase L levels (3- to 6-fold) as determined by covalently cross-linking of a 32P-labeled 2–5A analog to RNase L (Fig. 8D).

**DISCUSSION**

**Essential Role of RNase L in Viral Activation of JNK**—Our findings demonstrate that RNase L enhances viral activation of JNK. During EMCV infections there was a deficiency both in JNK activation and apoptosis in RNase L–/– cells compared with wild type cells. The possibility that epigenetic differences...
in the cell lines may have accounted for these findings was ruled out by reversal of phenotype because of induced expression of full-length but not truncated RNase L. Our results are also consistent with reports that RNase L is an important contributor to apoptosis in response to infections with a wide range of DNA and RNA viruses, including vaccinia virus and poliovirus (25, 41). Activation of JNK is a common cellular response to viral infections, including influenza virus, vesicular stomatitis virus, herpes simplex virus type I, and EMCV (see Refs. 1–3 and Fig. 1). JNK can potentially function in innate immune responses to viral infections through two distinct pathways, one transcriptional and the other through pre-existing apoptotic signaling factors. For instance, vesicular stomatitis virus induction of IFN-α and IFN-β promoters was severely deficient in Jnk2−/− cells (1). In addition, influenza A virus induction of the IFN-β promoter was dependent on JNK (2). On the other hand, sustained JNK activation leads to the mitochondria-mediated death pathway, involving Bcl2 family members Bak and Bax (11). Interestingly, JNK inhibition is known to increase viral yields compared with control-infected cells (2).

Viruses are believed to activate JNK, at least in part, through the viral pattern recognition molecule, dsRNA (1, 2). dsRNA is necessary for production of 2−5A oligoadenylates from ATP by the IFN-inducible 2−5A synthetases. 2−5A binds to RNase L, inducing formation of active dimers with potent endoribonuclease activity (24). A previous report demonstrated the requirement of RNase L for efficient JNK activation in response to dsRNA (20). However, dsRNA has profound effects on signaling pathways, other than the 2−5A/RNase L system, involving PKR, toll-like receptor 3, ADAR, and transcription factor IRF3 (42–45). In addition, we have found that it is possible to activate JNK with dsRNA even in cells lacking RNase L, although at lower levels than in wild type cells (data not shown). Therefore, it was important to show that RNase L could affect JNK activation by transfecting cells with 2−5A, the highly specific activator of RNase L. 2−5A activation of RNase L did indeed cause potent JNK activation in widely divergent cell types, mouse fibroblasts (MEF), and a human ovarian carcinoma cell line (Hey1b). JNK activation required the presence of the full-length, functional RNase L. In addition, 2−5A-mediated apoptosis was deficient in HeLa cells expressing an inactive missense mutant (R667A) form of RNase L. Results indicate a dose-dependent inhibition of protein synthesis inhibition up to the highest concentration of 2−5A tested (8 μM) (Fig. 2E). The 2−5A concentrations used in this study are within the physiologic range, for instance vaccinia virus-infected cells produce in the range of 5 μM 2−5A (46).

JNK Participation in RNase L-mediated Apoptosis—Three separate methods were used to demonstrate an essential role for JNK in the apoptotic signaling pathway induced by RNase L activation. Studies using a chemical inhibitor of JNK, siRNA

Fig. 6. The JNK inhibitor, SP600125, suppresses c-Jun phosphorylation and apoptosis in response to 2−5A activation of RNase L. A, c-Jun phosphorylation, PARP cleavage, and MTS assays (B) were performed on Hey1b cells treated the JNK inhibitor, SP600125, prior to 2−5A treatments. Pre-treatments with SP600125 were at the indicated concentrations (A) or at 25 μM (B) for 1 h prior to transfections with ps(A2′ps)5A. In A the times of treatment are shown to the right of the figure. In B the concentration of ps(A2′ps)5A was 4 μM. C, SP600125 does not inhibit RNase L. ps5′A(2′ps)5A (50 nM) and SP600125 (25 μM) were incubated with purified RNase L. Cleavage of a dual labeled RNA substrate by RNase L was determined by a FRET assay (see “Materials and Methods”). D, inhibition of p38 and ERK did not affect 2−5A-mediated apoptosis. Hey1b cells were treated with ERK inhibitor, 25 μM PD98059, or p38 inhibitors (2.5 μM SB202190 or 10 μM SB203580) for 1 h prior to mock transfections or transfections with 4 μM ps(A2′ps)5A for 1 h.

Fig. 7. Suppression of c-Jun phosphorylation and apoptosis by transfecting cells with siRNA against JNK1 and JNK2. Hey1b cells were transfected with 100 nM siRNA to JNK1/JNK2 mRNA for 24 h, before transfection of ps(A2′ps)5A at 4 μM for 16 h.
ablation of JNK, and JNK-null cells all demonstrated that loss of JNK function causes resistance to RNase L-mediated apoptosis. SP600125, a competitive inhibitor of ATP binding to JNK and some other kinases (39, 40), was able to greatly reduce apoptosis mediated by 2′–5A activation of RNase L. In addition, siRNA ablation of JNK1 and JNK2 reduced apoptosis to undetectable levels at 9 h post-2′–5A treatment. Apoptosis did occur after 24 h even in the presence of JNK1/JNK2 siRNA, possibly because of nonspecific induction of IFN (47). The most dramatic results were obtained by comparing wild type and Jnk1−/− Jnk2−/− MEFs. Apoptosis by the combination of IFN and 2′–5A, to induce and activate RNase L, respectively, was >7-fold higher in the wild type cells than in the JNK-null cells. On the other hand, inhibitors of alternative MAP kinases, p38 and ERK, did not affect apoptosis in response to RNase L activation. These findings show that RNase L activation results in a cellular stress response requiring JNK (but not p38 or ERK) for efficient induction of apoptosis.

The RNase L Apoptotic Signaling Pathway—The viral-induced pathway mediated through the 2′–5A/RNase L system is beginning to emerge from the present studies and prior published findings (Fig. 9). dsRNA produced during viral infections leads to 2′–5A synthesis and RNase L activation. Although the proximal RNA substrate(s) of RNase L that trigger JNK activation are unknown, rRNAs in intact ribosomes are candidates. Several different stimuli that target the peptidyl transferase ring or the adjacent S/R loop in 28 S rRNA require actively translating ribosomes to activate JNK (18). UVB, UVC, protein synthesis inhibitors blasticidin S and anisomycin, and enzymes that cleave (β-sarcin) and depurinate (ricin A chain) 28 S rRNA require actively translating ribosomes to activate JNK, termed the "ribotoxic stress response" (18). In comparison, RNase L cleaves 28 S rRNA in the L1 protuberance implicated in formation of the exit or E site of the ribosome, 324–326 bases 3′ to the β-sarcin and ricin cleavage/modification sites. Cleavage of 28 S rRNA at the RNase L susceptible site could possibly interfere with release of decayed tRNA (20). However, it is unlikely that JNK activation occurs solely as a result of protein synthesis inhibition, because several translation inhibitors, including emetine and pactamycin, completely inhibit protein synthesis without activating JNKs (18). In addition, pre-treatments of cells with cycloheximide or emetine did not prevent JNK activation by 2′–5A suggesting that the RNase L pathway to apoptosis occurs by a distinct mechanism compared with the ribotoxic stress response (Fig. 5 and data not shown).

![Fig. 8. Apoptosis in response to IFNα and 2′–5A is inhibited in Jnk1−/− Jnk2−/− MEF cells. A and B, Jnk1−/+ Jnk2−/+ and Jnk1−/− Jnk2−/− MEFs were transfected with 10 μg natural 2′–5A for the indicated times. A, in vitro c-Jun phosphorylation assay was performed in extracts of untreated and 2′–5A transfected cells. B, PARP cleavage was determined in Western blots assays. Levels of PARP cleavage are shown as a percentage. C, cells were pre-treated with or without 1,000 units per ml IFNα (BBDB) for 16 h prior to transfections with 10 μg natural 2′–5A. Apoptosis was determined by FACS TUNEL assay at 24 h post-treatment with 2′–5A. Results shown are an average of three separate treatments of cells with standard deviations. D, IFN-α treatment (1,000 units per ml for 16 h) induced RNase L levels as determined by covalent cross-linking of a 32P-2′–5A analog to RNase L (see “Materials and Methods”) (37). An autoradiogram of a dried gel is shown.](image-url)
analogs of 2–5A. Beihua Dong for mutant and wild type RNase L, Rune Hartmann for recombinant 2–5A synthetase, and Gregory Wroblewski and Malathi Krishnamurthy for preparing natural 2–5A.

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