Brief Definitive Report

A Study of the Interferon Antiviral Mechanism: Apoptosis Activation by the 2-5A System

By JoAnn C. Castelli,* Bret A. Hassel,‡ Katherine A. Wood,* Xiao-Ling Li,‡ Kei Amemiya,§ Marinos C. Dalakas,§ Paul F. Torrence,‖ and Richard J. Youle*

From the *Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892; ‡University of Maryland at Baltimore Cancer Center, Department of Microbiology and Immunology, Baltimore, Maryland 21201; §Neuromuscular Diseases Section, Medical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892; and ‖Section on Biomedical Chemistry, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

The 2–5A system contributes to the antiviral effect of interferons through the synthesis of 2–5A and its activation of the ribonuclease, RNase L. RNase L degrades viral and cellular RNA after activation by unique, 2₉–5₉-phosphodiester-linked, oligoadenylates [2–5A, (pp)p₅₋₉A₂₋₉(P₅₋₉A₂₋₉)n, n ≥ 2. Because both the 2–5A system and apoptosis can serve as viral defense mechanisms and RNA degradation occurs during both processes, we investigated the potential role of RNase L in apoptosis. Overexpression of human RNase L by an inducible promoter in NIH3T3 fibroblasts decreased cell viability and triggered apoptosis. Activation of endogenous RNase L specifically with 2–5A or with dsRNA, induced apoptosis. Inhibition of RNase L with a dominant negative mutant suppressed poly (I)·poly (C)-induced apoptosis in interferon-primed fibroblasts. Moreover, inhibition of RNase L suppressed apoptosis induced by poliovirus. Thus, increased RNase L levels induced apoptosis and inhibition of RNase L activity blocked viral-induced apoptosis. Apoptosis may be one of the antiviral mechanisms regulated by the 2–5A system.

Cells die by apoptosis during embryonic morphogenesis, tissue homeostasis, and in response to a number of chemotherapy and radiation regimens. Several lines of evidence suggest that apoptosis may also function as a host defense mechanism against viruses (1–3).

One of the most firmly established endogenous antiviral pathways is the 2–5A system which can be induced by virus infection and interferon treatment (4). Two of the key enzymes in the 2–5A pathway, 2–5A-dependent RNase (RNase L) and the family of 2–5’ oligoadenylate synthetases (OAS), are induced over constitutive basal levels after interferon treatment (5, 6). Upon viral infection, double-stranded RNA (dsRNA), apparently derived from viral replication intermediates, activates OAS resulting in the production from ATP of an unusual series of short 2–5’–linked oligoadenylates referred to as 2–5A (ppp₅’A₂₉p₅’A₂₉) (4, 6). As levels of 2–5A increase to nanomolar or higher concentrations, the latent RNase L is activated. RNase L binds specifically to 2–5A, cleaves single-stranded RNA with moderate specificity for sites 3’ of UpUp and UpAp sequences, and causes degradation of cellular rRNA (6, 7).

In view of the antiviral functions of both the 2–5A system and apoptosis, and the similar RNA degradation which occurs during both apoptosis (8, 9) and due to RNase L (10, 11), we explored the role of RNase L in cell death. We find that increasing RNase L expression or allosterically activating RNase L induces cell death whereas inhibiting RNase L activity protects cells from conditions associated with viral-induced apoptosis. RNase L thus appears to function in certain pathways of programmed cell death and initiation of apoptosis may be one mechanism for the antiviral activity of the 2–5A system.

Materials and Methods

For construction of inducible RNase L−stable transfectants, the lac repressor expression vector, p3’SS (Stratagene, La Jolla, CA), was transfected into NIH3T3 cells by calcium phosphate coprecipitation. Stable transfectants were selected in 250 µg/ml hygromycin and clonal cell lines were isolated. Lac repressor expression was measured on Western blots reacted to anti-Lac repressor antibody (Stratagene). Cell lines expressing high levels of Lac repres-
sor were transfected with the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible pOP13 plasmid containing the human RNase L cDNA in the sense orientation, cloned into the Not 1 site. Stable transfectants were selected in 500 μg/ml G418 and clonal cell lines were isolated for analysis.

For RNase L activity measurements, after indicated treatments, cells were washed with PBS and then incubated in serum-containing medium for an additional 3.5 h before harvesting. Total cellular RNA was isolated using Trizol (GIBCO BRL, Gaithersburg, MD) and 5 μg was electrophoresed on 1.4% agarose, stained with ethidium bromide, and visualized under UV fluorescence. Alternatively, RNA (10 μg) was separated by glyoxal-agarose gel electrophoresis and then transferred to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH) before hybridization to 32P-labeled 18S rRNA cDNA (12). The cDNA probe was radio-labeled with α-32P-deoxycytidine 5'-triphosphate using Prime-a-Gene system (Promega Corp., Madison, WI).

Cells were cultured overnight at 5 × 10⁴ cells/ml in Eagle’s Minimum Essential Medium (EMEM), containing 10% FCS, penicillin/streptomycin, gentamycin, and for stably transfected cells 200 μg/ml G418 (GIBCO BRL) and 250 μg/ml hygromycin. For protein synthesis inhibition assays, cells, in 96-well flat-bottomed plates, were incubated in leucine-free RPMI media containing 1% BSA, 0.1% saponin, and 1.5 mg/ml glycine. After 24 h of treatment, cells were twice washed and incubated in serum-containing media. Cells expressing GFP were counted at each of the indicated time points using fluorescent microscopy and calculated as a percentage of untreated GFP-positive cells (15).

**Results and Discussion**

Increased RNase L Expression Induces Apoptosis in Fibroblasts.

To investigate the potential role of RNase L in apoptosis, we generated NIH3T3 cells stably transfected with the human RNase L gene under the control of an IPTG-inducible lac promoter. One representative clone of seven independent clones characterized, 3T3/RNaseLS, expressed the human RNase L protein constitutively (Fig. 1 A, lane 2) and 24 h after induction with IPTG, expression levels increased by severalfold (Fig. 1 A, lane 3). To examine RNase L activity in the 3T3/RNaseLS cells, the RNase L activator, trimeric 2-5A [ppp5’(A2p5’)2A] was introduced into 3T3/RNaseLS cells. Specific 18S and 28S rRNA degradation products, characteristic of 2-5A-dependent RNase L cleavage (16), were detected in uninduced 3T3/RNaseLS cells (Fig. 1 B and C, lane 2) and this ribonuclease activity increased after IPTG induction (Fig. 1 B and C, lane 3). Similar results were found in two independent clones examined. Degradation of 28S rRNA, a characteristic of human RNase L (16), was not observed in 3T3/neo control cells expressing only the endogenous murine RNase L [data not shown] or in 3T3/RNaseLS cells induced by IPTG in the absence of 2-5A. Active human RNase L is expressed and inducible in 3T3/RNaseLS cells.

In the absence of IPTG, 3T3/RNaseLS cells grew more

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**Figure 1.** Inducible expression of human RNase L in NIH3T3 cells. (A) Western blot analysis was performed for the detection of human RNase L expression in vector control 3T3/neo cells (lane 1) and in NIH3T3 cells transfected with a lac inducible vector containing the human RNase L gene (3T3/RNaseLS) incubated 24 h in the absence (lane 2) or in the presence (lane 3) of 3 mM IPTG. Western blot analysis was performed as previously described (25) using a 1:2,500 dilution of a monoclonal antibody specific for the human RNase L enzyme which did not detect the endogenous murine RNase L (lane 1). Proteins were detected using ECL reagents (Amersham, Arlington Heights, IL). An autoradiograph of a 1-min exposure is shown. RNase L degradation after induction of RNase L (B) 3T3/RNaseLS cells were incubated in the absence (lanes 1 and 2) and presence (lane 3) of 3 mM IPTG for 24 h then transfected with 1 μM of ppp5’(A2p5’)2A (lanes 2 and 3) by calcium phosphate co precipitation as previously described (12). (C) Northern blot analysis using an 18S rRNA probe was shown after incubation of cells in the absence (lane 2) and presence (lanes 1 and 3) of 3 mM IPTG for 24 h followed by transfection with 1 μM of ppp5’(A2p5’)2A (lanes 2 and 3). Cellular RNA was isolated and electrophoresed as described in Materials and Methods.
staining for DNA fragmentation to 8.8–13.5% of the total RNase L expression for 48 h increased the number of cells. 

The 3T3/RNaseLS fibroblasts spontaneously underwent apoptosis at the characteristic DNA cleavage of apoptosis. The 3T3/neo (Fig. 3), we quantitated the number of cells that died with clones examined.

Similar results were found in two of two independent viabilities measured by Trypan blue dye exclusion to 38%. The rate to 20% of uninduced cells (Fig. 2) and decreased cell exposure to IPTG for 72 h decreased the protein synthesis of higher levels of RNase L in 3T3/RNaseLS cells by excluding dye were counted.

Using in situ DNA fragmentation detection (13, 14) (Fig. 3), we quantitated the number of cells that died with the characteristic DNA cleavage of apoptosis. The 3T3/RNaseLS fibroblasts spontaneously underwent apoptosis at a low rate (0–2.2%) and induction of higher levels of RNase L in 3T3/RNaseLS cells by exposure to IPTG for 72 h decreased the protein synthesis rate to 20% of uninduced cells (Fig. 2) and decreased cell viability measured by Trypan blue dye exclusion to 38%. Similar results were found in two of two independent clones examined.

Table 1. Growth rates of 3T3/neo and 3T3/RNaseLS Cell Lines

| Cell line    | Initial [3H]thymidine (cpm/well) incorporation rate ± SD | Cell density after 24 h (cells/well) |
|--------------|-----------------------------------------------|-------------------------------------|
| 3T3/neo      | 236 ± 30                                       | 2.8 × 10⁶                            |
| 3T3/RNaseLS  | 273 ± 54                                       | 1.6 × 10⁶                            |

* Cells were plated at 10⁴ cells/well and immediately after adherence they were washed twice and incubated in leucine-free RPMI media containing 0.5 μCi [3H]thymidine for 1.25 h. Cells were harvested onto glass fiber filters and radioactivity was determined by scintillation counting. * Cells were plated at 1.5 × 10⁵ cells/well and after 24 h of incubation, trypsinized, resuspended in Trypan blue dye, and cells excluding dye were counted.

10⁴-fold less effective as an activator due to the missing N1/N6 domain of the third adenine ring of parent 2-5A (18). These results correlate an increase in RNase L enzymatic activity with an induction in apoptosis.

Poly (I)-poly (C) has been shown to activate OAS, to increase levels of endogenous 2-5A, and to cause death of interferon-treated fibroblasts (6). We therefore examined whether poly (I)-poly (C) triggered apoptosis. Neither interferon nor dsRNA alone significantly reduced L929 cell viability or induced apoptosis (Fig. 4, A, B, D, and E). However, the combination of interferon and dsRNA caused an 88–91% decrease in cell viability and caused a large increase in the number of apoptotic cells (Fig. 4, C and F). To determine whether RNase L was required for this apoptosis pathway, as opposed to the interferon-inducible protein kinase (19), we transiently transfected L929 cells with a dominant negative inhibitor of RNase L. The inhibitor is a truncated version of RNase L, designated RNase L281, which lacks 89 COOH-terminal amino acids.

Table 2. Induction of A apoptosis by 2-5A

| L929 treatment* | Number apoptotic cells/Total | Percentage of apoptotic cell |
|-----------------|-----------------------------|-----------------------------|
| Mock transfected | 1/347                       | 0.3%                        |
| ppp2‘(A5’p2)2U transfected | 2/340 | 0.6%                         |
| ppp2‘(A5’p2)2A transfected | 90/333 | 27.0%                        |

* L929 cells were transfected by calcium phosphate coprecipitation for 75 min with a buffer control (mock), with 1 μM ppp2‘A5’p2‘A5’pA(2-5A). Cells were washed, incubated for 24 h, and then processed for apoptosis.

** Apoptosis was measured by counting cells in random fields positive for DNA fragmentation detected by TdT in situ labeling.

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Figure 3. RNase L overexpression caused DNA cleavage characteristic of apoptosis. 3T3/neo (A and C) and 3T3/RNaseLS (B and D) cells were incubated for 24 h in the absence (A and B) and presence (C and D) of 3 mM IPTG. Apoptotic cells were detected in situ by using T7 DNA polymerase with methyl green counterstaining. Experiments were performed in triplicate.

Figure 4. Apoptosis of interferon-treated fibroblasts after activation with dsRNA. In situ DNA end-labeling (A–C) and Hoechst dye no. 33342 staining (D–F) of L929 cells (A and D) After 48 h incubation in interferon-α/β (1,000 U/ml) (Sigma Chemical Co., St. Louis, MO). (B and E) 24 h incubation in synthetic dsRNA poly (I)·(C) (25 μg/ml). (C and F) 24 h preincubation in interferon-α/β followed by an additional 24 h in poly (I)-poly (C). Apoptotic cells were detected in situ by using terminal deoxynucleotidyl transferase with methyl green counterstaining. Cells were stained with Hoechst dye (0.1 mg/ml in PBS for 15 min) and photographed using fluorescence on an Axiovert microscope (Carl Zeiss, Inc., Thornwood, NY). Inhibition of RNase L protects fibroblasts against poly (I)-poly (C)-induced apoptosis. (G) L929 cells were transiently cotransfected with the GFP (green fluorescent protein) gene and either control vector (open circles) or the RNase LZB1 gene (closed squares) then incubated in interferon-α/β (1,000 U/ml) for 24 h. Cells were then treated with poly (I)-poly (C) (25 μg/ml) and green fluorescent cells were quantified as a measure of cell viability at each of the indicated time points. (25).
and lacks ribonuclease activity (12, 20). This truncated protein can function as a potent inhibitor of the catalytic activity of wild-type RNase L both in cell-free systems and in intact cells (12). Cells expressing the RNase L inhibitor remained viable significantly longer than the vector control cells after interferon and poly (I)-poly (C) treatment (Fig. 4 G). Thus, dsRNA can directly induce apoptosis in interferon-treated cells and inhibition of RNase L inhibits this cell death. Interestingly, transfection of cells with the Bcl-xL gene did not protect cells from poly (I)-poly (C)-induced apoptosis (Fig. 5 A). Either poly (I)-poly (C) induces an apoptosis pathway which is not blocked by Bcl-xL or it activates apoptosis downstream of the Bcl-xL regulatory point.

Inhibition of RNase L Blocks Poliovirus-induced Apoptosis. These results suggested that RNase L activity and the 2–5A pathway may mediate viral-induced apoptosis. We therefore examined poliovirus, a single-stranded RNA virus classified in the picornavirus family, which has been shown recently to induce apoptosis in HeLa cells (21). HeLa cells were transiently transfected with RNase LZB1, the dominant negative mutant, and then infected with poliovirus. After 45 h, only 22% of the vector control transfected HeLa cells remained viable, whereas 80% of the RNase LZB1 transfected cells remained viable (Fig. 6). Thus, inhibition of RNase L activity blocked apoptosis due to poliovirus. Interestingly, and in contrast to poly (I)-poly (C)-induced apoptosis, the poliovirus-induced cell death was blocked by transfection with the Bcl-xL gene (Fig. 5 B). Thus, RNase L activity was required for the poliovirus induced, Bcl-xL sensitive pathway of apoptosis.

RNase L was initially discovered as a mediator of the 2–5A dependent RNA breakdown stimulated by interferon and viral infections (4, 6). However, the precise mechanism for the antiviral effect of the 2–5A system in vivo is unknown. We show here that overexpression of RNase L triggers apoptosis, that allosteric activation of RNase L with 2–5A triggers apoptosis, and that a dominant negative inhibitor of RNase L blocks apoptosis induced by both dsRNA and poliovirus infection. This suggests that activation of RNase L by viral infection could serve to eliminate infected cells by apoptosis preventing viral spread through the cell population. This is a particularly intriguing hypothesis because several studies indicate that apoptosis is a cellular mechanism of viral defense; for example, virus infection itself can trigger apoptosis (22, 23), and many viruses express virulence factors which block apoptosis (24). The results presented here suggest that RNase L activates apoptosis of virus-infected cells to mediate the established antiviral activity of the 2–5A system.
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