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The role of 2′-5′ oligoadenylate-activated ribonuclease L in apoptosis

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Abstract

Apoptosis of viral infected cells appears to be one defense strategy to limit viral infection. Interferon can also confer viral resistance by the induction of the 2-5A system comprised of 2′-5′ oligoadenylate synthetase (OAS), and RNase L. Since rRNA is degraded upon activation of RNase L and during apoptosis and since both of these processes serve antiviral functions, we examined the role RNase L may play in cell death. Inhibition of RNase L activity, by transfection with a dominant negative mutant, blocked staurosporine-induced apoptosis of NIH3T3 cells and SV40-transformed BALB/c cells. In addition, K562 cell lines expressing inactive RNase L were more resistant to apoptosis induced by decreased glutathione levels. Hydrogen peroxide-induced death of NIH3T3 cells did not occur by apoptosis and was not dependent upon active RNAse L. Apoptosis regulatory proteins of the Bcl-2 family did not exhibit altered expression levels in the absence of RNase L activity. RNase L is required for certain pathways of cell death and may help mediate viral-induced apoptosis.

Keywords: programmed cell death; interferon; viruses; staurosporine; diethylmaleate

Abbreviations: OAS 2′-5′, oligoadenylate synthetase; RNase L, 2′,5′ A-dependent RNase; 2-5A, 2′-5′ linked oligoadenylates; ICE, interleukin-1β converting enzyme; rRNA, ribosomal RNA; CML, chronic myelogenous leukemia

Introduction

Apoptosis is an active process distinguishable from necrosis by characteristics including nuclear condensation, cellular shrinkage, DNA fragmentation, and rapid phagocytosis (Wyllie, 1980). Tumor suppressors, oncogenes and proteases are among the myriad of factors implicated in apoptosis but none have provided a defined biochemical function as it pertains to cellular survival.

Cells frequently undergo apoptosis after viral infection. Due to the discovery of numerous viral-expressed apoptosis inhibitors it has been proposed that apoptosis may function as a host defense mechanism against viruses (Clem *et al*, 1991; Clouston and Kerr, 1985). Although some viruses trigger apoptosis and eliminate specific cell populations, more prevalent is the induction of viral factors to block cellular apoptosis providing increased virulence and persistent infection. For example, crmA is a poxvirus protein that blocks apoptosis by interacting with ICE, a cysteine protease homologous to Ced3 (Gagliardini *et al*, 1994), and the E7 protein of some papilloma virus strains binds to and inactivates p53 in a ubiquitin-dependent pathway. Unknown mechanisms account for the antiapoptotic actions of several of the RNA viruses including picornaviruses, alphaviruses, and retroviruses, that may express specific proteins to prevent apoptosis (Tolskaya *et al*, 1995). The array of viral factors that block cellular apoptosis and are also required for virulence strongly supports the model that apoptosis is a cellular host defense system against viral infection.

Another cellular anti-viral strategy is the interferon induced 2-5A pathway, a well-established system that confers protective and anti-proliferate properties on both normal and transformed cells. The development of the antiviral state requires interferon induction of the 2′,5′ oligoadenylate synthetase (OAS) and the 2′,5′ A-dependent RNase (RNase L) (Dong *et al*, 1994). Viral infection appears to generate double-stranded RNA species that stimulate OAS to produce 2′→5′ phosphodiester linked oligoadenylates (2-5A) which activate RNase L. Activated RNase L subsequently cleaves both cellular and viral messenger RNA and generates a distinct degradation pattern of rRNA fragments. The degradation of rRNA is directly associated with the activation of the 2-5A system, by either 2-5A, interferon or EMC virus infection (Silverman *et al*, 1983).

Furthermore, rRNA degradation has been observed to coincide with apoptosis following gamma-irradiation (Houge *et al*, 1995) and cAMP activation (Houge *et al*, 1993). Considering that rRNA degradation occurs during apoptosis and viral infection, we examined the role RNase L plays in cell death. We show here that eliminating RNase L activity protects cells from a number of conditions that induce apoptosis. RNase L appears to function in several pathways of programmed cell death.

Results

Dominant negative mutant cells lack RNase L expression and activity

To investigate the role of the 2-5A system in apoptosis, cell lines were examined in which RNase L activity was either

blocked or deficient. The RNase L inhibitor is a truncated version of RNase L, called RNase L_{ZB1}, that lacks 89 C-terminal amino acids and lacks ribonuclease activity (Hassel *et al*, 1993; Zhou *et al*, 1993). 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 (Hassel *et al*, 1993). RNase L_{ZB1} was stably expressed in murine SV40-transformed BALB/c cells (SVT2), in murine NIH3T3 cells and in human K562 cells. The relative levels of RNase L and RNase L_{ZB1} protein in the transfected and control cells were measured by crosslinking with radiolabeled 2-5A (Nolan-Sorden *et al*, 1990) (Figure 1A). It has been previously documented that SVT2/ZB1 cells express RNase L_{ZB1} and lack RNase L activity (Hassel *et al*, 1993). The 3T3/ZB1.4 cell line is a clonally selected transfectant that has extinguished RNase L_{ZB1} expression and shows a 40-fold reduction in endogenous RNase L enzyme levels (Figure 1A, lane 3) compared to vector control cells (Figure 1A, lane 2). The 3T3/ZB1.4 cells contain barely detectable amounts of RNase L and provide an important model cell line for studying the involvement of RNase L activity in apoptosis, as any unintended influence of overexpressing the dominant negative inhibitor is absent. A stable clone of the K562 cells, K562/ZB1 (cl. 30) expressed 240-fold higher levels of truncated RNase L (Figure 1A, lane 5) as compared to the wild-type enzyme in vector control K562 cells (Figure 1A, lane 4). K562/ZB1 (cl. 35) cells, which failed to express the truncated RNase L (Figure 1A, lane 6), served as an additional negative control.

The degree of inhibiton of RNase L activity was measured (Figure 1B). Upon introduction of trimeric 2-5A into the 3T3/neo cells in the absence of interferon, characteristic 18S rRNA cleavage products were detected (Figure 1B, lane 3). Pretreatment of 3T3/neo cells with murine interferon ($\alpha+\beta$) to elevate levels of RNase L and 2′,5′ oligoadenylate synthetase (Figure 1B, lane 2) resulted in higher levels of 18S cleavage products. However, 3T3/ZB1.4 cells did not exhibit 18S rRNA degradation (Figure 1B, lanes 4–6), with or without interferon pretreatment. Therefore, there was no measurable RNase L activity in the 3T3/ZB1.4 cells. Similar results have been published for SVT2/ZB1 cells expressing the truncated RNase L compared to the SVT2/pSVL control cells (Hassel *et al*, 1993). In the human CML-derived K562 cell line, introduction of trimeric 2-5A induced cleavage of 18S rRNA in the vector control K562/neo cells, (Figure 1B, lane 8) whereas no breakdown of 18S rRNA was observed in the K562/ZB1 (cl. 30) cells (Figure 1B, lane 10), which overexpresses RNase L_{ZB1}. Thus, endogenous RNase L activity is functionally inhibited in SVT2/ZB1 and K562/ZB1 (cl. 30) cells and deficient in 3T3/ZB1.4 cells.

Inhibition of RNase L blocks staurosporine-induced apoptosis

Staurosporine, a broad specificity protein kinase inhibitor, has been shown to induce apoptosis in human fibroblasts (Jacobson *et al*, 1994) and other cell lines (Bertrand *et al*, 1994). We evaluated the role of RNase L in cell death in BALB/c and NIH3T3 fibroblasts. We confirmed that a 24 h exposure to 1 μM staurosporine-caused apoptosis in NIH3T3 cells by staining with Hoechst no. 33342 dye and by *in situ* detection of DNA fragmentation (Gavrieli *et al*, 1992; Wood *et al*, 1993). Suppression of RNase L activity dramatically blocked cell death due to staurosporine (Figure 2A and B). Both the SVT2 cells expressing RNase L_{ZB1} (SVT2/ZB1) and the NIH3T3 cells lacking RNase L (3T3/ZB1.4) required more than a tenfold greater concentration of staurosporine to induce cell death compared to vector control cells (Figure 2A and B). We examined cell death with trypan blue dye exclusion, at various times after exposure to 1 μM staurosporine (Table 1) and found that SVT2/ZB1 and 3T3/ZB1.4 cell lines were resistant to staurosporine-induced apoptosis. Thus, cell lines lacking RNase L activity are more resistant to staurosporine-induced apoptosis as compared to cell lines with active RNase L.

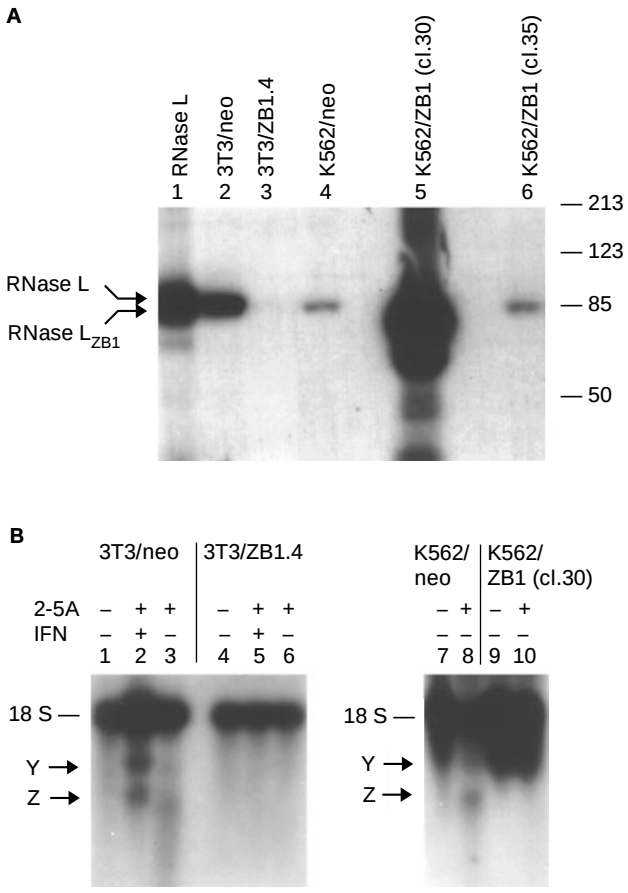

Figure 1 RNase L and dominant negative inhibitor (RNase L_{ZB1}) levels in NIH3T3 and K562-derived cell lines. (**A**) Truncated and wild-type RNase L proteins were specifically radiolabeled by covalent crosslinking to a ^{32}P-labeled and bromine substituted 2-5A probe, $p(A2'p)_2(br^8A2'p)_2A3'$-[^{32}P]Cp. Human recombinant RNase L in a baculovirus vector produced in insect cells and labeled with the 2-5A probe is shown (lane 1). Names of cell lines and the position of RNase L, RNase L_{ZB1} and the molecular weight markers (in kDa) are indicated. (**B**) RNase L activity is blocked in cells expressing RNase L_{ZB1} as determined by analysis of 18S rRNA after 2-5A transfection with or without interferon (1000 U/ml) treatment. Degradation products following activation of RNase L are indicated. Cells were plated overnight then transfected with 4 μM of $ppp5'(A2'p5')_2A$ by calcium phosphate co-precipitation for NIH3T3 cells or with 300 μg/ml DEAE-dextran for K562 cells

Staurosporine-induced apoptosis is independent of protein synthesis

We examined whether the RNase L deficient cell lines were resistant to other effects of staurosporine. In both SVT2 and NIH3T3 cell lines, protein synthesis in cells lacking (SVT2/ZB1 and 3T3/ZB1.4) and expressing (SVT2/pSVL and 3T3/neo) RNase L activity was equally inhibited by staurosporine (Figure 2C and D). Thus, the two RNase L deficient cell lines have similar cytosolic sensitivity to staurosporine yet fail to initiate apoptosis. This result also shows that *de novo* protein synthesis does not appear to be required for the RNase L-dependent induction of apoptosis. Consistent with this observation, pretreatment of 3T3/neo cells with 10 μg/ml cycloheximide did not inhibit the staurosporine-induced apoptosis.

Inhibition of RNase L decreases nuclear condensation characteristic of apoptosis

When the morphology of 3T3/ZB1.4 cells was examined with Hoechst staining after 24 h exposure to staurosporine, the nuclei appeared normal (Figure 3D) whereas vector control

Table 1 Time course of staurosporine-induced apoptosis*

Time (h)	pSVL/SVT2	pSVL/ZB1	3T3/neo	3T3/ZB1
6	45	82	102	117
10	5.0	22	41	110
24			1.0	75

*At each of the indicated timepoints following 1 μM staurosporine treatment, cell viability, as a percent of naive controls, was measured by trypan blue dye exclusion

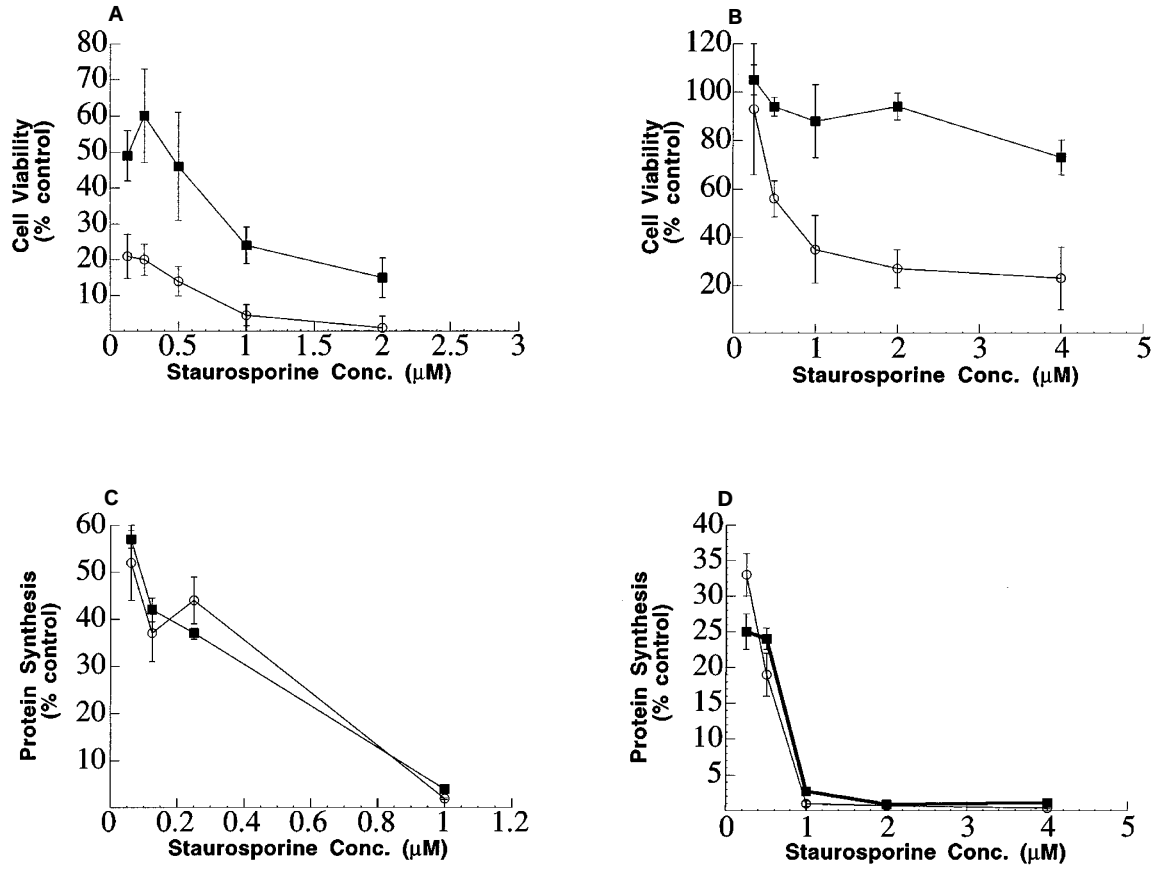

Figure 2 Inhibition of endogenous RNase L blocks apoptosis of 3T3 cells. (**A**) SV40 transformed BALB/c 3T3 cells transfected with control vector (SVT2/pSVL) (circles) and with vector expressing truncated RNase L (SVT2/ZB1) (squares) and (**B**) NIH3T3 cells with native levels of RNase L (3T3/neo) (circles) and cells deficient in expression of RNase L (3T3/ZB1.4) (squares) were evaluated for cell viability by trypan blue dye exclusion after 24 h exposure to varying concentrations of staurosporine. Cells were cultured overnight at 5×10^4 cells/ml in EMEM, containing 10% FCS, penicillin/streptomycin, gentamycin and 500 μg/ml G418 (Gibco BRL) and then incubated in staurosporine (Sigma) at the indicated concentrations for 24 h. Protein synthesis was measured after 24 h exposure to staurosporine in (**C**), SVT2/pSVL cells (circles) and SVT2/ZB1 cells (squares) and in (**D**) 3T3/neo cells (circles) and 3T3/ZB1.4 cells (squares). Cells were incubated overnight in EMEM, 10% FCS, supplemented with 500 μg/ml G418, and for 3T3/RNaseLS cells, 250 μg/ml hygromycin at 5×10^4 cells/ml in 96 well flat-bottomed plates. After 24 h treatment, cells were incubated in leucine-free RPMI media containing 0.5 μCi ^{14}C leucine for 1.25 h. Cells were harvested onto glass fiber filters and radioactivity determined by scintillation counting. Experiments were performed in duplicate and plotted $\pm$ standard deviation

cells displayed condensed nuclei (Figure 3B). The number of apoptotic cells in 3T3/ZB1.4 cells after staurosporine treatment was only 2% compared to 98% in vector control cells. Thus, decreasing RNase L activity prevents the morphological change in nuclei and the loss in cell viability by staurosporine.

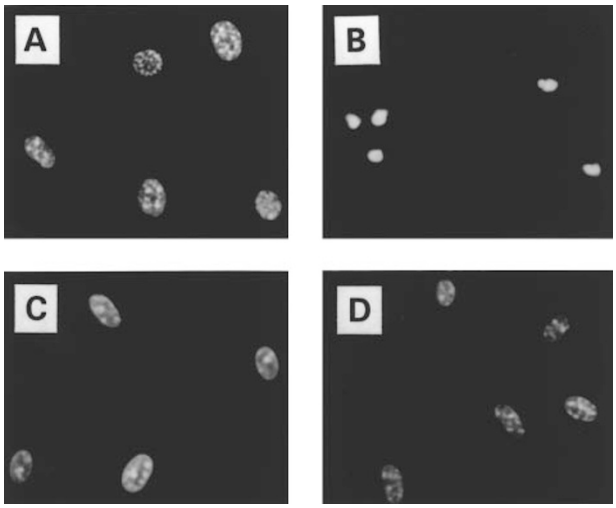

Figure 3 Nuclear condensation of NIH3T3 cells caused by staurosporine is not observed in the absence of RNase L expression. 3T3/neo cells (**A**, **B**) and 3T3/ZB1.4 cells (**C**, **D**) were incubated in the absence (**A**, **C**) and presence (**B**, **D**) of 1 μM staurosporine for 24 h and nuclear morphology was evaluated by Hoechst dye no. 33342

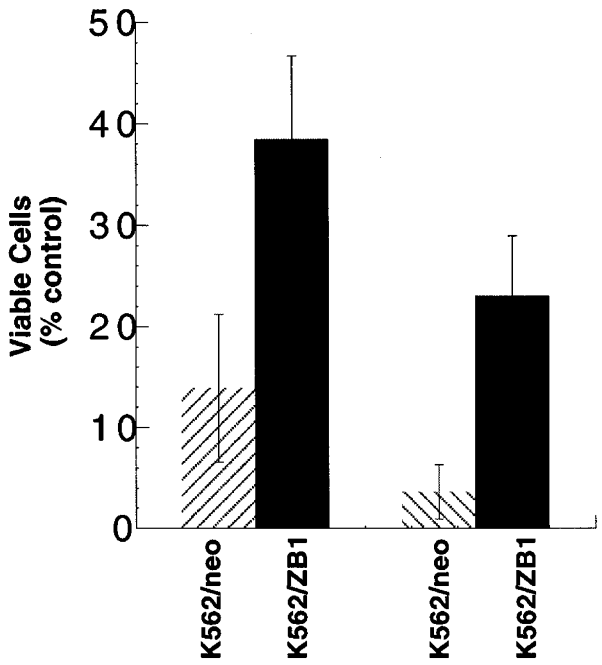

Figure 4 Cell viability of K562 cells stably transfected with vector control (hatched bars) and RNase L_{ZB1} (darkly shaded bars) following 1 mM (left two bars) or 4 mM (right two bars) diethylmaleate exposure to decrease glutathione levels. Cells were incubated 24 h with or without DEM and cell viability, as a percent of DEM naive controls, was determined by trypan blue dye exclusion

Inhibition of RNase L blocks DEM-induced apoptosis

Diethylmaleate (DEM) decreases glutathione levels (Ku and Billings, 1986) and triggers apoptosis in GT1-7 (Kane *et al*, 1993) and in K562 cells (data not shown). We evaluated the involvement of RNase L in apoptosis by comparing the sensitivity of K562/ZB1 cells to DEM with that of control K562 cell lines. K562 cells lacking RNase L activity were resistant to apoptosis induced by DEM (Figure 4).

Hydrogen peroxide-induced cell death

Hydrogen peroxide is an oxidizing agent that can induce apoptosis (Hockenbery *et al*, 1993) or necrosis (Schraufstatter *et al*, 1986) depending upon the type of cell treated. Examination of the nuclear morphology after 24 h exposure to

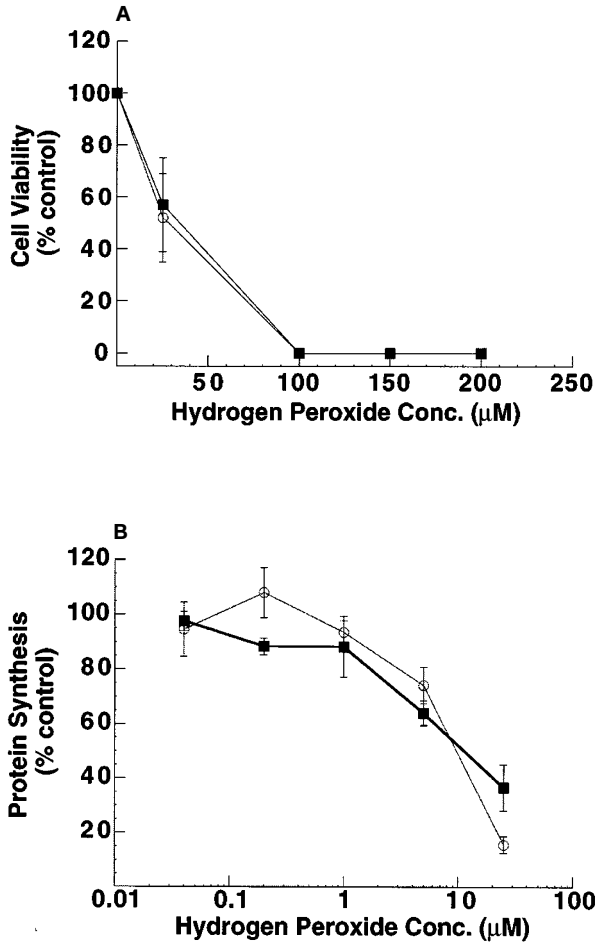

Figure 5 NIH3T3 cells lacking RNase L are not resistant to hydrogen peroxide-induced cell death. (**A**) 3T3/neo cells containing RNase L activity (open circles) and 3T3/ZB1.4 cells lacking RNase L activity (closed squares) were evaluated by trypan blue dye exclusion at each of the indicated doses. Cell viability with H_2O_2 treatment is shown as the percentage of untreated cells. (**B**) Protein synthesis was measured in 3T3/neo cells, (open circles), and 3T3/ZB1.4 cells, (closed squares) after 24 h incubation with various concentrations of H_2O_2 and shown as a percent of untreated cells. Mean was computed $\pm$ standard deviation of duplicate cultures

moderate doses of H_2O_2 that killed 50% of the cells with Hoechst dye revealed that NIH3T3 cells do not have the classical apoptotic morphology of nuclear condensation and fragmentation (data not shown). Similar results were found at higher doses of H_2O_2 (200 μM) that killed greater than 95% of the cells. NIH3T3 cell lines lacking and expressing RNase L were incubated in media containing varying concentrations of hydrogen peroxide, ranging from 25–200 μM. The two cell lines were almost identical in their rates of cell death in response to each of the concentrations (Figure 5A), indicating that RNase L is not required for cell death induced by hydrogen peroxide. Thus, hydrogen peroxide-induced fibroblast cell death, that does not occur by apoptosis, does not require RNase L activity. Examination of protein synthesis in the peroxide-treated cells showed identical inhibition in the same dose range as that used to induce cell death (Figure 5B). Thus the similarity in rates of cell death and protein synthesis due to oxidation-induced necrosis contrasts with Figure 2, which illustrates a distinction between the cellular response to staurosporine-induced apoptosis and inhibition of protein synthesis.

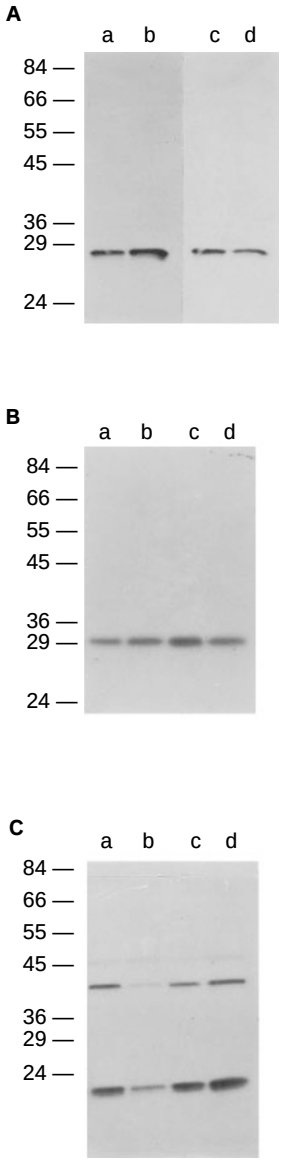

Figure 6 Expression of Bcl-2, Bcl-X_L and Bax in various transfected cell lines. Solubilized cell lysates of pSVL/SVT2 (a), and pSVL/ZB1 (b), 3T3/neo (c), 3T3/ZB1.4 (d), were subjected to electrophoresis over 12% SDS polyacrylamide gels and the proteins were transferred onto Immobilon membranes for labeling with either anti-Bcl-2 polyclonal antibody (**A**), anti-Bcl-X_L monoclonal antibody 2H12 (**B**), or anti-Bax polyclonal antibody (**C**)

Bcl-2, Bcl-x_L and Bax expression

Ribonucleases can regulate gene expression by regulating RNA stability (Shaw and Kamen, 1986) and this has been suggested to be one physiological function of RNase L. We find RNase L is required for at least one pathway of apoptosis and investigated whether RNase L regulates the expression of proteins known to induce or block apoptosis. Bcl-2 (Garcia *et al*, 1992; Hockenbery *et al*, 1990) and Bcl-x_L (Boise *et al*, 1993) can block apoptosis and Bax (Oltvai *et al*, 1993; Sato *et al*, 1994) can increase the rate of apoptosis. We examined the protein levels of Bcl-2, Bcl-x_L and Bax in wild-type and dominant negative RNase L mutant cell lines (Figure 6). Bcl-2 and Bcl-x_L protein levels remain unchanged in the presence and absence of RNase L in both NIH3T3 and SV40 transformed cell lines (Figure 6A and B). Bax protein levels are unchanged in the NIH3T3 cell lines but are slightly lower in the RNase L deficient SV40 transformed cell line in comparison to the vector control (Figure 6C). This indicates that the role of RNase L in apoptosis is not to alter mRNA levels that result in destabilizing Bcl-2, Bcl-x_L or stabilizing Bax.

Discussion

Our current findings provide a possible explanation for many previous studies linking 2-5A synthetase activity, RNA breakdown or ribonuclease activation to cell death or tissue regression. (Cidlowski, 1982; Delic *et al*, 1993; Houge *et al*, 1993, 1995; Maor, 1962; Perreault and Lemieux, 1993; Weymouth, 1958). The regression of chick oviduct following withdrawal of estrogen was accompanied by an increase in 2-5A synthetase activity (Stark *et al*, 1979), an increase in levels of 2-5A *per se* and the breakdown of ovalbumin mRNA and 18S rRNA (Cohrs *et al*, 1988). Similarly, 2-5A and related oligoadenylates were observed in rat mammary glands that were regressing during cessation of lactation (Reid *et al*, 1984). The cytocidal effect of treating the HT29 human colon carcinoma with both TNF-α and interferon-γ was accompanied by an increase in 2-5A synthetase levels and apparent rRNA breakdown (Chapekar and Glazer, 1988). Furthermore, total RNase activity in the spleen and the thymus dramatically increased with gamma-irradiation (Maor, 1962; Weymouth, 1958), a potent inducer of apoptosis in lymphocytes and glucocorticoids, that also induce lymphocyte apoptosis, were found to stimulate RNA degradation in rat lymphocytes (Cidlowski, 1982). A latent RNase is activated during pupation of fly larvae, a time of massive apoptosis during metamorphosis (Aoki and Natori, 1981). More recently rRNA degradation has been found to occur simultaneously with

apoptosis induced by a number of agents (Delic *et al*, 1993; Houge *et al*, 1993, 1995). This rRNA degradation is reminiscent of the rRNA cleavage induced by RNase L including the selective cleavage in uridine-rich regions in both systems. In B cells, several mRNAs are degraded concurrently with DNA fragmentation during apoptosis (Perreault and Lemieux, 1993). As apoptosis is a physiological cell removal pathway that does not trigger an inflammatory response, cell catabolism could be postulated to be an important function of the pathway. Thus cells may self-digest many of their components to minimize body exposure to cellular debris. In this light it may not be surprising that RNases or DNases may be activated during this process. However, our results showing that cells lacking RNase L activity fail to die indicate a more essential role of RNase L in apoptosis.

Our results extend the known biological role of RNase L beyond the interferon system. Evidence presented here showing the participation of RNase L in nonviral pathways of apoptosis 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 consistent with recent findings showing that RNase L is required for viral-induced apoptosis (Castelli *et al*, 1997; Diaz-Guerra *et al*, 1997). RNase L thus appears to participate in host antiviral apoptosis to mediate the established antiviral activity of the 2-5A system.

RNase L has been proposed to function physiologically in the regulation of mRNA turnover and thus RNase L may mediate apoptosis by regulating the expression of other proteins essential to the apoptosis pathway. The presence or absence of RNase L in two different cell systems did not significantly affect the levels of three proteins, Bcl-2, Bcl-x_L, and Bax, that regulate apoptosis. However, RNase L may regulate other proteins involved in apoptosis.

Our results indicate that RNase L plays a more essential, causal role in cell death induced by viral (Castelli *et al*, 1997; Diaz-Guerra *et al*, 1997) and nonviral stimuli and represents an important new step in the apoptosis cascade.

Materials and Methods

Construction of stably transfected cell lines

For dominant negative stable transfectants, truncated murine RNase L cDNA (RNase L_{ZB1}) (Hassel *et al*, 1993) was cloned into the *Not*I and blunt-ended *Xba*I sites of the expression vector pcDNAIneo (Invitrogen) in the sense orientation. Supercoiled plasmid (10 μg) was transfected into 5×10^5 NIH3T3 cells by calcium phosphate coprecipitation. Stable transfectants were selected by growth in medium containing 500 μg/ml G418 and surviving cells were clonally isolated. K562 cells were transfected by electroporation; 5×10^6 cells were transfected with 10 μg supercoiled plasmid at 400 V, 800 μF. Stable transfectants were selected by growth in 250 μg/ml G418 and individual clones were isolated by limiting dilution. Control cells, 3T3/neo and K562/neo, were transfected with pcDNAIneo vector alone. SVT2 cells expressing the truncated RNase L were generated as described (Hassel *et al*, 1993).

Assay of truncated and endogenous, wild- type RNase L using a bromine substituted ^{32}P-labeled 2-5A analogue

Truncated and wild-type RNase L were specifically radiolabeled by covalent crosslinking to a ^{32}P-labeled and bromine-substituted 2-5A probe, $p(A2'p)_2(br^8A2'p)_2A3'$-[^{32}P]Cp (Nolan-Sorden *et al*, 1990). Cells were washed in phosphate buffered saline, harvested by scraping, and used to prepare cell extracts as described previously (Nolan-Sorden *et al*, 1990). About 4×10^5 counts/min. of the 2-5A probe, at about 3000 Ci/mmole, was incubated with cell extracts (300 μg of protein per sample) on ice for 1 h and then crosslinking was performed for 1 h on ice under ultraviolet light at 308 nm. Protein separation was by SDS/10% polyacrylamide gel electrophoresis and was followed by autoradiography. Quantitation of 2-5A binding activity was by phorphorImage analysis (Molecular Dynamics) of the dried gels.

RNase L activity assay in intact cells

Cells were seeded at 6×10^5 cells per 100 mm plate for 24 h and were transfected with 4 μM of $p_3(A2'p5')_2A$ by calcium phosphate coprecipitation (for NIH3T3 and SVT2 cells) or with 300 μg per ml DEAE-dextran (for K562 cells). Cells were incubated with $p_3(A2'p5')_2A$ for 75 min in low serum-containing medium (Opti-MEM, Gibco BRL). In some experiments, NIH3T3 cells were pretreated for 16 h with 1000 U/ml of murine interferon (α/β) (5×10^7 U/mg: Lee Biomolecular) prior to 2-5A transfection. Cells were washed with phosphate buffer saline and then incubated with serum-containing medium for an additional 3.5 h before harvesting. Total cellular RNA was isolated using the RNazol reagent (Tel-Test, Friendswood, TX).

RNA (30 μg) was separated by glyoxal-agarose gel electrophoresis (Zhou *et al*, 1993) and then transferred to Nytran membrane and hybridized to ^{32}P-labeled 18S rRNA cDNA (provided by Dr. John Thaden, Baltimore). The cDNA probe was radiolabeled with α-^{32}P-deoxycytidine 5′-triphosphate using Prime-a-Gene system (Promega Co.).

Cytotoxicity assays

Cells were cultured at a density of 2×10^6 cells per 4 ml EMEM, 10% fetal calf serum, antibiotics, and 500 μg/ml G418 in 35 mm plates (Falcon). Staurosporine (Sigma) in DMSO at 500 μM was serially diluted in PBS before addition to cells in 8 μl to achieve the desired final concentration. All cells treated with staurosporine received less than 0.5% DMSO and were compared to appropriate DMSO controls. Floating and adherent cells were centrifuged and the number of viable cells was determined by trypan blue dye exclusion. For protein synthesis inhibition assays, cells (5×10^5 cells/ml) were plated in 200 μl media EMEM in 96 well plates for 24 h at varying concentrations of the indicated agent, washed in RPMI 1640 leucine free media then incubated for 1 h in leucine free, serum free RPMI 1640 media containing 0.5 μCi ^{14}C leucine. Cell protein was collected on glass fiber filters with a Ph.D cell harvester (Cambridge Technology, Inc.) and the radioactivity determined by scintillation counting.

K562 cells were plated at a density of 2×10^6 cells in 4 ml RPMI 1640, 10% fetal calf serum, antibiotics and 100 μg/ml G418 in 35 mm plates. Diethylmaleate (Sigma) diluted in sterile H_2O was added to cells at each of the final concentrations indicated. Viable cells were assessed by trypan blue dye exclusion.

Apoptosis detection

NIH3T3 cells, plated on poly-L lysine coated glass cover slips in 24 well plates, were incubated in serum-free media then treated for 5 min at room temperature with (0.1 mg/ml in PBS) Hoechst dye no. 33342 (Sigma). K562 cells, plated in 35 mm dishes (Falcon), were incubated in the appropriate treatment for 24 h, then similarly stained with Hoechst dye. *In situ* staining of cells for DNA fragmentation was performed as reported previously (Gavrieli *et al*, 1992; Wood *et al*, 1993). Cells were grown on poly-L lysine-coated glass coverslips in 24 well plates in serum-free media and incubated with and without staurosporine 24 h before analysis of apoptosis.

Western blotting analysis of Bcl-2, Bcl-X_L and Bax expression levels

For immunoblots, 5×10^6 cells from either 3T3/neo, 3T3/ZB1, SVT2/pSVL, or SVT2/ZB1 cultures were solubilized in 200 μL of 10 mM HEPES, pH 7.4, 90 mM KCl, 10 μg/ml leupeptin, and 1% IGEPAL CA-630 and 20 μg protein was electrophoresed on a 12% SDS polyacrylamide gels. The proteins were electroblotted onto Immobilon membranes. The membranes were blocked in phosphate buffered saline (PBS) containing 0.05% Tween-20 and 5% nonfat milk for 1 h. The membranes were then incubated with either α-Bcl-2 polyclonal antibody (Santa Cruz; 1 : 100), α-Bax rabbit polyclonal antiserum (Krajewski *et al*, 1994) (1 : 300) or α-Bcl-X_L monoclonal antibody 2H12 hybridoma culture fluid in the blocking buffer for 45 min. The membranes were then washed in PBS containing 0.05% Tween-20 and relabeled with either donkey anti-rabbit or sheep α-mouse immunoglobulin peroxidase (Amersham Corp.; 1 : 3000) in the blocking buffer for 30 min. After washing with the Tween/PBS buffer, the labelings were visualized by ECL (Amersham Corp).

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