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(2′-5′)A_n-Dependent Endoribonuclease: Enzyme Levels Are Regulated by IFNβ, IFNγ, and Cell Culture Conditions

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The levels of a (2′-5′)A_n-dependent endonuclease (RNase L) were determined in extracts prepared from murine L cells and Ehrlich ascites tumor (EAT) cells by measuring specific binding of protein to a labeled derivative of (2′-5′)A_n, (2′-5′)A_3[^{32}P]pCp. RNase L levels were found to depend both on interferon (IFN) treatment and on cell growth conditions. Treatment of murine L cells and EAT cells with 100–2,000 IRU IFNβ or IFNγ resulted in a similar 2–4-fold increase in the levels of RNase L when cells were present at low density. The levels of RNase L were also shown to increase 2–3-fold as cells approached saturation density. Serum-starved cells also displayed relatively high levels of RNase L. RNase L levels in cells maintained at high cell density did not change appreciably following treatment with IFNβ or IFNγ. Regulation of RNase L levels by cell growth conditions as well as by IFNβ or IFNγ treatment suggests that RNase L may play an important role in regulating the levels of cellular mRNAs as well as acting to degrade viral RNAs.

Key words: interferon β, interferon γ, (2′-5′)oligoadenylates, RNase L, anti-oncogenes

Interferons are antiviral proteins [see 1, for review] that may also regulate cell growth rates [2] and the immune response [3,4]. Three types of interferon (IFN) proteins, IFNα, IFNβ, and IFNγ [5], and the genes encoding them have been identified and extensively characterized [see 1,6 for review]. IFNα and IFNβ bind to the same cell surface receptor and are functionally indistinguishable [6]. IFNγ binds to a different cell surface receptor and appears to have functions that are not shared with IFNα/β [6–8].

Some of the effects of IFNα/β are mediated by IFN-induced proteins that are activated by double-stranded RNA (dsRNA) [1]. These enzymes block virus replication at the level of mRNA degradation [1,9]. A key enzyme in the RNA degradative pathway

Abbreviations used: IFN, interferon; (2′-5′)A_n, n = 2 to 15, 5′-triphospho-oligo(2′-5′)adenylyl- (2′-5′)adenosine; (2′-5′)A_3pCP, 5′-triphospho-(2′-5′)adenylyl-(2′-5′)adenylyl-(2′-5′)adenylyl-(3′-5′)cytidine phosphate (^{32}P-labeled); dsRNA, double-stranded RNA; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; RNase L, a (2′-5′)A_n-dependent endonuclease; EAT, Ehrlich ascites tumor cells, IRU, NIH interference reference units.

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is a latent endonuclease (RNase L) that is activated by (2′-5′)oligoadenylates [[(2′-5′)]A_n] [10]. (2′-5′)A_ns are synthesized by another IFNα/β-induced enzyme, the (2′-5′)$A_n$synthetase [11]. The effects of IFNα/β on the levels of RNase L have been characterized in some cells [10,12–15]; however, effects of IFNγ have not been reported previously. IFNγ is known to induce the (2′-5′)$A_n$synthetase; however, induction is decreased compared to IFNα/β and shows different induction kinetics [16]. A decrease in cell growth rate is also known to induce both RNase L [13–15] and the (2′-5′)$A_n$synthetase [17,18], suggesting that these enzymes may be involved in regulation of cell growth rates as well as the antiviral response to IFN.

In the studies presented here, experiments further characterizing IFNβ-mediated as well as IFNγ-mediated regulation of RNase L levels in murine cell lines are described. Modulation of RNase L levels in murine L cells and in EAT cells by cell culture conditions is also discussed.

METHODS

Cell Culture, Experimental Treatment Protocols, and Cell Extract Preparation

L cells (B. Jacobs, Arizona State University) and EAT cells (American Type Culture Collection, Rockville, MD) were maintained in media containing 5% fetal bovine serum. Cells were subcultivated at a 10–20-fold dilution into 100-mm^2 tissue culture dishes, treated with IFNβ or IFNγ, or mock IFN as indicated in legends, harvested, and counted with a hemocytometer. Murine IFNβ (5.6×10^7 IRU/mg), mock IFN (> 2 IRU/mg), and rabbit anti-IFN α/β immunoglobulin were from Lee Biomolecular (San Diego, CA). Recombinant murine IFNγ (1.9×10^7 IRU/mg) was a gift from Dr. Michael Shepard at Genentech (San Francisco, CA). IFN activity was assayed as described [19]. Extracts were prepared as described [20] except that 1 mM phenylmethylsulfonyl fluoride (PMSF) was present. Two fractions, a postmitochondrial supernatant (cytoplasmic extract) and a crude nuclear pellet containing primarily nuclei and other large organelles, were stored at −70°C. A nuclear extract was prepared from crude nuclei as described previously [20]. Protein concentrations were determined according to the Bradford method (Biorad Inc., Richmond, CA) [21].

Assays for RNase L

Endonuclease activity was ascertained by using rRNA as a substrate as described previously [12–15]. The protocol for binding of RNase L to a labeled derivative of (2′-5′)A_3, [^{32}P](2′-5′)A_3pCp (S.A. 3,000 Ci/mmol) in a complex that is retained on nitrocellulose filters has been described [20]. Extracts were always thawed on ice to avoid inactivation of RNase L [22]. Photo-crosslinking of RNase L to (2′-5′)A_3pCp and analyses of the labeled proteins were performed essentially as described [20,23].

RESULTS

The (2′-5′)A_3pCp Binding Assay in Cell Extracts

The (2′-5′)A_3pCp binding assay is a quantitative measure of RNase L levels in cell extracts and in fractions collected during purification of RNase L [20]. (2′-5′)A_3pCp binding activity and (2′-5′)A_n-dependent RNase activity copurify [20,23] and both activities are associated with a single polypeptide (Mr 80,000) [10,20]. The (2′-5′)A_3pCp

binding assay is the most quantitative measure of the levels of RNase L [20] currently being used and is the primary assay used here. In order to make comparisons of RNase L levels in extracts, conditions required for optimal, linear (2′-5′)A_3pCp binding activity were established (data not shown) and consistently applied.

Proteolytic degradation can complicate quantitative determinations of protein amounts and has been previously reported to occur in extracts assayed for RNase L [10,14]. Proteolysis of RNase L (Mr 80,000) is readily observed using an assay in which (2′-5′)A_3pCp acts as an affinity label for RNase L [12–15]. A small labeled protein (Mr 45,000) is apparently a degradation product of RNase L. Formation of this small protein can be prevented by addition of protease inhibitors during preparation of extracts [14] and/or centrifugation (30,000g) of extracts prepared by dounce homogenization [20]. Cytoplasmic extracts prepared by the latter method appear to have low protease activity, which may be due to removal of organelles, i.e., lysosomes, from the cytoplasm during centrifugation. In order to compare proteolytic activity in cytoplasmic and crude nuclear pellet extracts, these were crosslinked to (2′-5′)A_3pCp and analyzed by polyacrylamide gel electrophoresis and autoradiography [20,23]. Figure 1 (lanes A and B) shows an autoradiogram of proteins crosslinked to (2′-5′)A_3pCp from cytoplasmic extracts [10,20,23]. One cytoplasmic protein (Mr 80,000) contained almost all of the crosslinked (2′-5′)A_3pCp. Figure 1 (lanes A′, B′, C′) shows an autoradiogram of crosslinked proteins present in the crude nuclear extract. In contrast to cytoplasmic extracts, crude nuclear extracts prepared in the absence of protease inhibitor (Fig. 2, lane C′) contained primarily a small protein (Mr 40,000–45,000) crosslinked to (2′-5′)A_3pCp with little, if any, of a larger protein (Mr 80,000). Preparation of nuclear extracts in the continuous presence of fresh 1 mM PMSF prevented proteolytic degradation of the large protein (Fig. 1, lanes A′ and B′). Highly purified nuclei do not contain significant protease activity [23], suggesting that the protease responsible for degrading RNase L may be associated with other organelles contaminating the crude nuclear preparations.

RNase L Levels in Murine Cells Lines

RNase L levels as determined by using the (2′-5′)A_3pCp binding assay vary considerably depending upon the cell type and culture conditions [10,12–15]. Because cell culture conditions are known to influence RNase L levels in some cell types, the effects of cell density, media replenishment, and serum starvation were determined for both L cells and EAT cells. RNase L levels in EAT cells were compared during log phase growth and as cells approached saturation density (Fig. 2). These data show that at low cell densities the levels of RNase L are about one-fourth to one-third that seen in extracts from cells harvested at high density. Removal of depleted media and addition of fresh media resulted in higher cell density but lower levels of (2′-5′)A_3pCp binding activity. This suggests that RNase L levels in EAT cells increase concurrently with growth arrest associated with nutrient depletion of media. The role of serum concentration in regulation of RNase L levels was further explored by determining RNase L levels in cells grown in media partially depleted in fetal bovine serum (Table I). Serum starvation resulted in an overall lower rate of cell growth and an overall decrease in cell density. Cells grown in high fetal calf serum (6%, 8%, and 10%) attained higher cell density (0.80–1.0 $\times$ 10^6 cells/ml) than did those maintained in low fetal calf serum (0.3–0.5 $\times$ 10^6 cells/ml). RNase L levels, however, did not rise appreciably in cells grown with low serum. This observation suggests that RNase L levels in EAT cells are regulated,

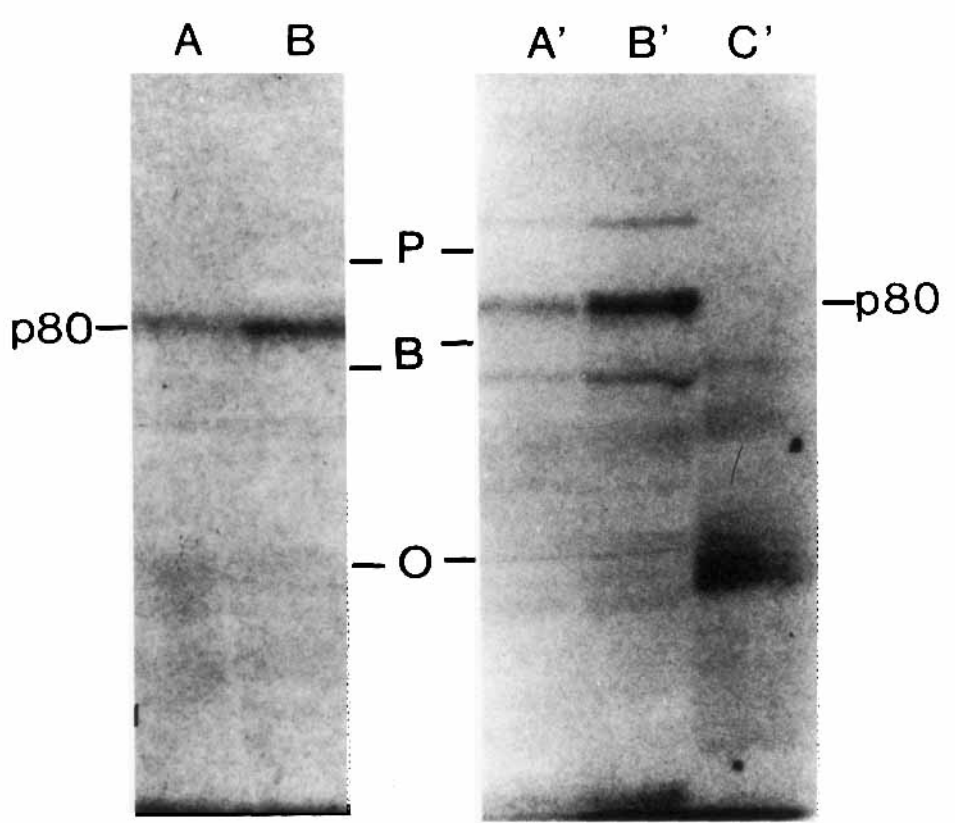

Fig. 1. Photo-crosslinking of (2′-5′)A_3pCp to protein present in cytoplasmic and nuclear extracts from control and IFNβ-treated EAT cells. Aliquots of cytoplasmic or nuclear cell extracts (200 μg) were incubated in 0.1-ml assay mixtures containing 125,000 cpm (2′-5′)A_3[^{32}P]pCp and photo-crosslinked by using ultraviolet light (254 nm) as previously described [20]. Proteins were separated by SDS polyacrylamide gel electrophoresis and labeled proteins were visualized by autoradiography. The mobility of protein standards, phosphorylase a (Mr 92,500) (**P**); bovine serum albumin (Mr 68,000) (**B**); and ovalbumin (Mr 45,000) (**O**), as well as a protein (Mr 80,000) photo-crosslinking to (2′-5′)A_3[^{32}P]pCp, (p80), are indicated. Incubation mixtures contained cytoplasmic extract (**A**, **B**) or nuclear extract (**A′**, **B′**, **C′**) from EAT cells prepared as previously described [23]. Extracts prepared in the presence of 1 mM PMSF from cells pretreated for 20 hr with 1,000 U IFNβ at low cell density are shown in lanes B and B′ and from control cells are shown in lanes A, and A′. Protein photo-crosslinked to (2′-5′)A_3[^{32}P]pCp from a nuclear extract prepared in the absence of PMSF is shown in lane C′.

not only by cell growth rates, but also perhaps by some other factor associated with high cell density.

RNase L levels are also regulated in murine L cells by cell culture conditions. Cytoplasmic extracts of L cells harvested at high cell density generally contained twice the amount of RNase L activity (25–35 fmol (2′-5′)A_3pCp bound/mg) as those harvested at low cell density (10–15 fmol (2′-5′)A_3pCp bound/mg). Serum starvation of L cells was associated with relatively high levels of RNase L, as shown in Table I. This suggests that RNase L levels in L cells are regulated somewhat differently than in EAT cells and that the increase in RNase L levels depends primarily on cell growth rates.

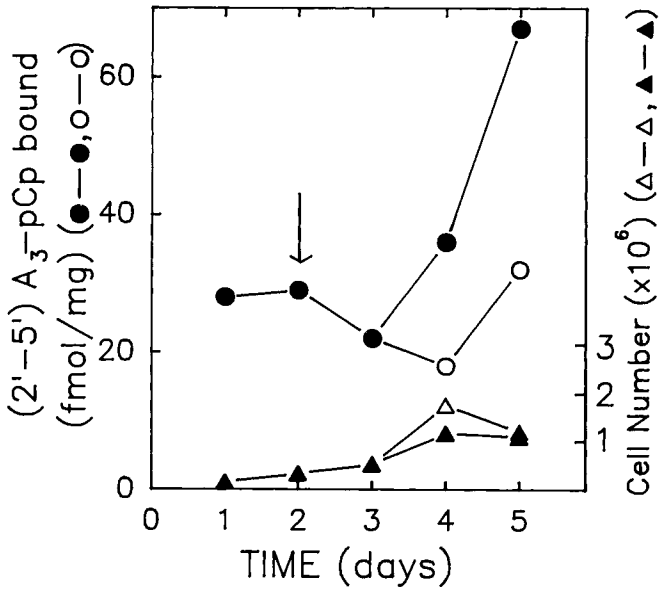

Fig. 2. (2′-5′)A_3[^{32}]pCp binding activity in EAT cells maintained in culture for 1–5 days with or without media replenishment. EAT cells were grown to high density, diluted in fresh media, and subcultured into 100 mm^2 tissue culture dishes. Cells were collected at the times indicated and the cell number determined (triangles). Protein concentrations and (2′-5′)A_3pCp binding activity (circles) were determined as described under Materials and Methods. The arrow indicates addition of fresh media to some cultures (○,△)whereas others (●,▲) did not undergo a change in media.

TABLE I. RNase L Levels as a Function of Fetal Bovine Serum Concentration*

Fetal bovine serum (%)	(2′-5′)A_3pCp bound (fmol/mg)	
	EAT cells	L cells
1	—	23.7 ± 2.2
2	13.7 ± 3.1	23.2 ± 0.5
4	12.4 ± 4.6	29.2 ± 9.2
6	26.8 ± 4.7	26.9 ± 5.2
8	23.0 ± 4.0	—
10	23.7 ± 4.5	—

*EAT cells and L cells were subcultivated into tissue culture dishes containing the indicated amounts of fetal bovine serum and were harvested 3 days after subcultivation. Cytoplasmic extracts were prepared and assayed for (2′-5′)A_3pCp binding activity as described under Materials and Methods. The means and standard deviations of four replicate experiments are given.

The Effect of Interferon Treatment on RNase L Levels in Murine Cells

RNase L is a key enzyme mediating some of the antiviral actions of IFN [1,10]. RNase L levels increased in cytoplasmic and nuclear extracts of both EAT and L cells in response to IFNβ treatment (Fig. 1, Tables II and III) with maximal induction following IFNβ treatment of 18–22 hr (data not shown).

Important structural and functional differences between IFNβ and IFNγ have been well documented [1–4,6]. Because of these differences, it was of interest to determine

TABLE II. Effect of Interferon Treatment on the Levels of (2′-5′)A_3pCp Binding Activity in Murine EAT Cells

Treatment	Cell density ($\times 10^6$ cells/ml)	(2′-5′)A_3pCp binding activity[a] (fmol/mg)	% control
Exp. 1			
Control	1.01	38.9	—
100 IRU IFNβ	.95	34.2	88
500 IRU IFNβ	.94	40.1	103
1,000 IRU IFNβ	.87	40.1	103
2,000 IRU IFNβ	.66	41.8	107
Exp. 2			
Control	.48	14.5	—
100 IRU IFNβ	.41	34.0	230
500 IRU IFNβ	.30	42.7	294
1,000 IRU IFNβ	.32	56.3	388
Exp. 3			
Control	.81	26.3	—
100 IRU IFNγ	.96	29.1	110
500 IRU IFNγ	.59	26.1	99
1,000 IRU IFNγ	.67	26.1	99
Exp. 4			
Control	1.01	23.0	—
100 IRU IFNγ	.98	31.1	135
1,000 IRU IFNγ	.65	21.5	93
2,000 IRU IFNγ	.81	31.7	138
Exp. 5			
Control	.21	9.8	—
1,000 IRU IFNγ	.16	37.3	380

[a]Binding activity is given as the average of duplicate samples.

whether IFNγ, like IFNβ, regulated RNase L levels. Table II shows a comparison between the levels of RNase L in control EAT cells and in cells treated with IFNβ or IFNγ at different cell densities. EAT cells growing at low cell densities showed a 3–4-fold increase in RNase L levels following treatment with murine IFNβ. Extracts prepared from EAT cells growing at very low densities responded to IFNγ treatment as well. Treatment of cells growing at high density with IFNβ or IFNγ had little effect on RNase L levels. The levels of RNase L in IFNβ- or IFNγ-treated L cells (Table III) were also determined. Addition of comparable amounts of IFNβ or IFNγ resulted in a 1.5–3-fold increase in the levels of RNase L in these cells when cells were actively growing at low cell density. IFN treatment resulted in an overall decrease in cell number as expected [2].

Spontaneous IFN production has been previously observed in some cell types [24] and has been thought to contribute to the rise in RNase L levels seen in cells collected at high density. This possibility was investigated. Media supernatants assayed for antiviral activity [19] were found to contain less than 1 IRU/ml antiviral activity. Addition of rabbit anti-mouse IFNα/β immunoglobulin failed to prevent the increase in RNase L levels associated with high density (data not shown). This suggests that regulation of RNase L occurs through an IFN-independent mechanism as well as an IFN-dependent mechanism.

TABLE III. Effect of Interferon Treatment on the Levels of (2′-5′)A_3pCp Binding Activity in Murine L Cells

Treatment	Cell density ($\times 10^6$ cells/ml)	(2′-5′A_3pCp binding activity[a] (fmol/mg)	% control
Exp. 1			
Control	1.44	23	—
100 IRU IFNβ	.88	23	100
1,000 IRU IFNβ	.44	22	95
Exp. 2			
Control[b]	2.15	28.5	—
100 IRU IFNβ +	2.25	37.2	131
1,000 IRU IFNβ +	1.11	35.1	123
Exp. 3			
Mock IFN	.25	14.0	—
20 IRU IFNβ	.30	15.0	107
100 IRU IFNβ	.24	25.0	178
1,000 IRU IFNβ	.13	29.0	207
Exp. 4			
Control	1.55	11.5	—
100 IRU IFNγ	1.52	15.3	133
1,000 IRU IFNγ	1.12	12.6	109
Exp. 5			
Control	1.21	13.9	—
500 IRU IFNγ	1.14	21.5	155
1,000 IRU IFNγ	.98	23.1	166
2,000 IRU IFNγ	1.06	23.9	172
Exp. 6			
Control	.84	8.0	—
100 IRU IFNγ	.74	12.1	151
1,000 IRU IFNγ	.38	20.5	256
Exp. 7			
Control	.36	8.0	—
300 IRU IFNγ	.35	21.7	271
1,000 IRU IFNγ	.30	20.4	255
2,000 IRU IFNγ	.31	28.4	355

[a]Binding activity is given as the average of duplicate samples.
[b]Medium was removed and replaced with fresh medium at the time of IFN addition.

DISCUSSION

Levels of RNase L in cytoplasmic cellular extracts are influenced by several factors including cell type, culture conditions, and treatment with IFNβ or IFNγ. Differential proteolytic degradation of RNase L does not account for observed differences in cytoplasmic extracts prepared from L cells and EAT cells prepared following treatment with IFNβ, IFNγ or growth of cells under various conditions. Extracts prepared from crude nuclei, on the other hand, seem to contain protease activity that generates a protein of 40,000–45,000 daltons from one of about 80,000 daltons which specifically crosslinks to (2′-5′)A_3pCp. The protease(s) which is located in a particulate subcellular fraction is inactivated by PMSF. It is not known whether this protease plays a functional role in regulating RNase L protein levels in vivo.

Regulation of cell growth rates involves the action of many hormones, growth factors, and intracellular growth-regulatory proteins [25]. Some proteins, having homology to viral oncogenes, stimulate cell growth [25,26]. Other proteins may be required for normal, regulated cessation of cell growth [25]. Interferons appear to function as negative cell growth regulators [2,27], and it has also been suggested that enzymes of the (2′-5′)$A_n$synthetase-RNase L pathway may be intracellular mediators of this process [27–29]. The cleavage specificity of RNase L does not preclude a role for this enzyme in regulation of cellular mRNA, hnRNA, and/or rRNA levels [30]. If RNase L does play a role in cessation of cell growth, then the observed rise in enzyme levels concurrent with cell growth arrest is expected.

Established cell lines such as L cells or EAT cells, unlike normal cells, are not subject to normal mechanisms regulating cell division and have very rapid cell growth rates. This rapid rate of division and the high saturation density attained by these cells occur in the presence of relatively high levels of RNase L. This suggests that if RNase L is involved in regulation of cell growth, the normal regulatory mechanism may be overridden by the oncogenic processes taking place in these cells. Further experiments designed to compare regulation of RNase L levels in normal cells, tumor cells, and in murine tissues have been initiated to explore this hypothesis.

RNase L levels increase in cells treated with IFNβ or with IFNγ at comparable antiviral units. The increase in RNase L levels following IFN treatment is greatest when cells are present at low cell density and are rapidly growing at the time of IFN addition. Cytoplasmic RNase L is regulated similarly by both IFNβ and IFNγ. This is somewhat surprising because expression of a (2′-5′)$A_n$synthetase gene is regulated differently by IFNα/β and IFNγ [28,29] and because others [12,15] have failed to observe regulation of RNase L levels by IFNγ.

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