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Interferon-γ activates the cleavage of double-stranded RNA by bovine seminal ribonuclease

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Bovine seminal ribonuclease (BS-RNase), a dimeric homologue of RNase A, cleaves both single- and double-stranded RNA and inhibits the growth of tumor cells. Its catalytic activity against double-stranded RNA, either homopolymeric ([^{3}H]polyA/polyU) or mixed sequence, is enhanced by bovine or human recombinant interferon-γ (IFN-γ). Activation is seen with as little as 4–10 interferon units per assay. Enhancing the degradation of double-stranded RNA, an intermediate in the growth cycle of many viruses, could contribute to IFN-γ's ability to control cell growth and induce an antiviral state.

Interferon-γ; Interferon-α; Ribonuclease A (RNase A); Bovine seminal ribonuclease; Mechanism of cell growth inhibition by interferons; Activation of double-stranded RNA degradation

1. INTRODUCTION

Increased synthesis or translation of specific messenger RNAs in cells treated by interferons (IFN) [1] is considered to be the most probable mechanism for the induction by interferon of an antiviral state and its inhibition of cell growth. However, recent evidence has indicated that interferons may also influence the stability [2,3] and processing [4] of mRNAs. As interferons also inhibit angiogenesis in vitro [5] and in vivo [6] and as factors capable of inducing angiogenesis include a ribonuclease homologue (angiogenin) and, possibly, ribonucleoproteins [7], we have undertaken a study of the interaction between interferons and ribonucleases.

2. MATERIALS AND METHODS

We first found that IFN-γ preparations stimulate the catalytic activity of the non-digestive RNase from bovine seminal fluid (BS-RNase) (but not RNase A or B), when single-stranded RNA is the substrate [8]. Bovine seminal ribonuclease has a relatively higher activity on double-stranded (ds) RNA substrates than RNase A. We show here that IFN-γ stimulates even more the activity of BS-RNase, but not of RNase A, when the substrate in the nuclease assay is homopolymeric (polyA/polyU) or mixed sequence double-stranded (ds) RNA. Activation of mixed sequence ds-RNA cleavage is observed at concentrations (1–500 IFN units/ml, 1–2 nM [9]) that are physiologically relevant.

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Abbreviations: IFN-γ, interferon-γ; IFN-α, interferon-α; hu, human; bo, bovine; RNase A, ribonuclease A; BS-RNase, bovine seminal ribonuclease; IFU, interferon units; ss- or ds-RNA, single-stranded or double-stranded RNA; TCA, trichloroacetic acid

3. RESULTS AND DISCUSSION

The catalytic activity of BS-RNase against homopolymeric ds-RNA [^{3}H]polyA/polyU) is increased by human recombinant IFN-γ, while the activity of RNase A is either unaffected or, at high concentrations, inhibited by IFN-γ (Table I). This inhibition, which is also seen at very high concentrations of IFN-γ in the assays of single- and double-stranded mixed sequence RNA, is probably related to the fact that IFN-γ binds RNA tightly [8]. (This binding can also be seen in Fig. 2 when high concentrations (>200–300 IFU/assay or >1 μg/ml of the human IFN) are added.)

When human IFN-γ is preincubated with monoclonal or polyclonal antibodies that neutralize its activity in antiviral assays and inhibit its binding to cell-surface receptors, its ability to activate BS-RNase is lost (Table II). Antibodies (such as monoclonal antibody γ-127) which bind but do not neutralize IFN-γ do not lower the ability of IFN-γ to activate BS-RNase. Monoclonal antibody γ-69, which is about 100-fold more inhibitory

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Table I

Comparison of the effect of recombinant human IFN-γ on the rate of hydrolysis of double-stranded RNA catalyzed by RNase A and bovine seminal ribonuclease

Addition	Percentage ds RNA solubilized	Percentage control activity
Buffer	13	
Buffer plus IFN-γ, 0.5 mg/ml	11	
BS-RNase, 0.2 mg/ml	91	
(1) RNase A, 0.1 mg/ml	61	(100)
idem + human IFN-γ at:		
$5 \cdot 10^{-1}$ mg/ml	37	50
$5 \cdot 10^{-2}$ mg/ml	67	112
$5 \cdot 10^{-3}$ mg/ml	56	89
(2) BS-RNase, 0.02 mg/ml	22	(100)
idem + human IFN-γ at:		
$5 \cdot 10^{-1}$ mg/ml	57	489
$5 \cdot 10^{-2}$ mg/ml	75	689
$5 \cdot 10^{-3}$ mg/ml	21	100
(3) BS-RNase, 0.002 mg/ml	17	(100)
idem + human IFN-γ at:		
$5 \cdot 10^{-1}$ mg/ml	20	175
$5 \cdot 10^{-2}$ mg/ml	43	750
$5 \cdot 10^{-3}$ mg/ml	16	100

Like interferon isolated from natural sources, the human IFN-γ used lacks Cys-Tyr-Cys at the N-terminus. IFN-$\gamma\Delta$ Cys (1 μl of the indicated dilution) was combined with 1 μl of BS-RNase or RNase A (diluted to the indicated concentration in BSA/Tris), 5 μl of 'ds-RNase assay mix' was added, the samples incubated 1 h at 37°C, the reactions stopped with the addition of TCA and carrier RNA and protein, and the pellet collected by centrifugation as described [8]. [^{3}H]PolyA/polyU was prepared by combining 50 μl ($\approx$40 pmol) [^{3}H]polyadenylic acid ($n \approx 50$) with 0.8 mg polyuridylic acid (Pharmacia) in 200 μl H_2O. The sample was heated to 80°C for 15 min and then cooled slowly to room temperature. The 'ds-RNase assay mix' contained 30 μl of the annealed substrate in 500 μl of Tris buffer, pH 7.9, 25 mM, which gave about 1250 cpm per assay (dissolved in Rotiszint 22 (Roth)). Note the buffer control (13% of the total RNA is acid soluble due to the inhomogeneity of the substrate) must be substracted from all numbers to determine the percentage RNA solubilized by the enzyme. 'pecentage control activity' is the activity of the sample containing IFN-γ relative to that of the nuclease alone

Table II

Antibodies to human IFN-γ neutralize their activation effect on bovine seminal ribonuclease in the percentage RNA solubilized assay using double-stranded RNA [^{3}H]polyA/polyU)

Addition	Percentage RNA solubilized	Percentage control
Buffer control	15	
BS-RNase plus buffer	30, 30	(100)
BS-RNase +:		
no antibody, γ diluted in PBS-BSA	42, 44	187
monoclonal 127, 35 μg (non-neutralizing)	44, 48	207
monoclonal 123, 31 μg (weakly neutralizing)	34, 35	133
monoclonal 69, 40 μg (γ-neutralizing)	29, 28	93
affinity-purified polyclonal, 43 μg (γ-neutralizing)	35, 39	147

IFN-γ (250 ng, diluted with 1 mg/ml macaloid treated (to remove ribonucleases [11] BSA in PBS) was added to PBS/BSA buffer (control) or to 10 μl of antibody in PBS (containing the indicated amount of protein), the samples were incubated for 5 h at 4°C and centrifuged in an Eppendorf centrifuge for 15 min. For the assay, 2 μl of a $5 \cdot 10^{-3}$ mg/ml solution of BS-RNase in PBS/BSA were combined with 2 μl of the preincubated IFN-γ containing solution, the ds-RNA nuclease assay mix was added (see Table I), the samples were incubated 1 h at 37°C, and stopped by addition of TCA and carrier protein and RNA. None of the antibody solutions alone contained appreciable nuclease activity in this assay nor did they activate the nuclease. Antibodies (in PBS) were obtained from G. Garotta of Roche Research, Basel; neutralization of IFN-γ activity was determined by inhibition of IFN-γ activity in the antiviral assay (in Zürich) and its binding to cells (in Basel). Note the buffer control (15% of the total RNA is acid soluble due to the inhomogeneity of the substrate) must be substracted from all numbers to determine the percentage RNA solubilized by the enzyme. 'Percentage control' is the activity of the sample containing IFN-γ relative to that of the nuclease alone (based on the average of duplicate samples)

than γ-123 in the antiviral assay, also inhibits the activation of BS-RNase more than γ-123. Both of the neutralizing antibodies bind to human IFN-γ near residue 80 (Garotta, G., personal communications). Monoclonal antibody γ-127 binds to a basic region of IFN-γ near the C-terminus.

Recombinant human IFN-α2, human interleukin-2 and murine IFN-α have no effect on the activity of BS-RNase on either single- or double-stranded RNA substrates (data not shown). Nor does lysozyme, another positively charged protein, have a marked effect. At very high concentrations lysozyme partially inhibits the cleavage of ss-RNA but not the rate of cleavage of ds RNA (Table III). Lysozyme, like IFN-γ, binds RNA tightly at high concentrations (data not shown), and we believe this accounts for the RNase inhibition observed.

IFN-γ also activates BS-RNase and inhibits RNase A when the substrate in the nuclease reaction is heterosequence double-stranded (ds) RNA. This is presumably a biologically more relevant substrate than the homopolymer. Indeed, a nuclease active on double-stranded RNA substrates was recently reported to be co-induced with IFN-γ [10]. Both human (Figs. 1b and 2b) and bovine (Figs.1b and 2c) IFN-γ markedly increase the rate of cleavage of ds-RNA by BS-RNase. In contrast, both bovine and human IFN-γ inhibit the cleavage of ds-RNA by RNase A (Figs. 1a and 2a), an inhibition that correlates well with the binding of the substrate ds-RNA by the IFN-γ's (Fig. 2a).

With either substrate approximately a 3–10-fold molar excess of interferon-γ must be added to activate BS-RNase. Relatively more BS-RNase must be used to degrade the homopolymeric substrate than the mixed

Table III

Comparison of the effect of recombinant human interferon-γΔ Cys with that of another positively charged protein, hen egg lysozyme (Sigma), on the activity of bovine seminal ribonuclease against single-stranded (ss-RNA) and double-stranded (ds-RNA) RNA

Addition:	Percentage ss-RNA solubilized	Percentage ds-RNA solubilized
Buffer	2,3	11
BS-RNase plus buffer	54, 47 (100)	33, 31 (100)
BS-RNase + human IFN-γ at:		
0.5 mg/ml	65 (127)	54, 75 (255)
$5 \cdot 10^{-2}$ mg/ml	67 (131)	65, 47 (214)
$5 \cdot 10^{-3}$ mg/ml	69 (135)	24, 22 (57)
$5 \cdot 10^{-4}$ mg/ml	70 (137)	not done
BS-RNase + lysozyme at:		
1 mg/ml	10 (20)	26 (71)
10^{-1} mg/ml	41 (80)	43, 34 (131)
10^{-2} mg/ml	68 (133)	30, 34 (100)
10^{-3} mg/ml	62 (122)	25, 27 (71)
10^{-4} mg/ml	45 (88)	not done

The proteins (1 μl of the indicated dilution in BSA/Tris) were combined with 1 μl of a solution of BS-RNase ($2 \cdot 10^{-4}$ mg/ml in BSA/Tris) and assayed for ss-RNase activity as described in Fig. 1 or with 1 μl of a 10^{-2} mg/ml solution of BS-RNase and assayed for activity on [^{3}H]polyA/polyU (ds-RNA) as described in Table I. Some samples were simultaneously duplicated, as indicated. The numbers in parentheses indicate the percentage RNA solubilized relative to that solubilized by the nuclease alone

sequence ds RNA; consequently, more IFN-γ is needed to show activation. Approximately the same activation was seen whether the buffer was low salt (Tris, 25 mM, used in most experiments) or relatively high salt (PBS, Table II).

These data are interesting for several reasons. First, the RNase activation reported here is the first assay for the activity of an interferon that does not require mammalian cell culture. As little as 3–4 IFN units can be detected if the gel assay of Fig. 2 is used, making it as sensitive as the most commonly used antiviral assay. While the antiviral assay takes 2–3 days, the RNase stimulation assay takes 2–3 h. Of course, the presence of nucleases in many biological fluids means that the assay is only possible with at least partially purified samples of interferon.

Further, antibodies that block the biological activity of IFN-γ in vivo inhibit the ability of IFN-γ to activate BS-RNase in vitro, and the activity of antibodies that block the activation of BS-RNase by IFN-γ correlates with their ability to neutralize the activity of IFN-γ in two different mammalian cell assays. Of course, many viruses produce ds-RNA at some point in their growth cycle. The ability to bind nucleic acids and to regulate the activity of molecules involved in their degradation, demonstrated in this study, may be involved in the biological function of IFN-γ.

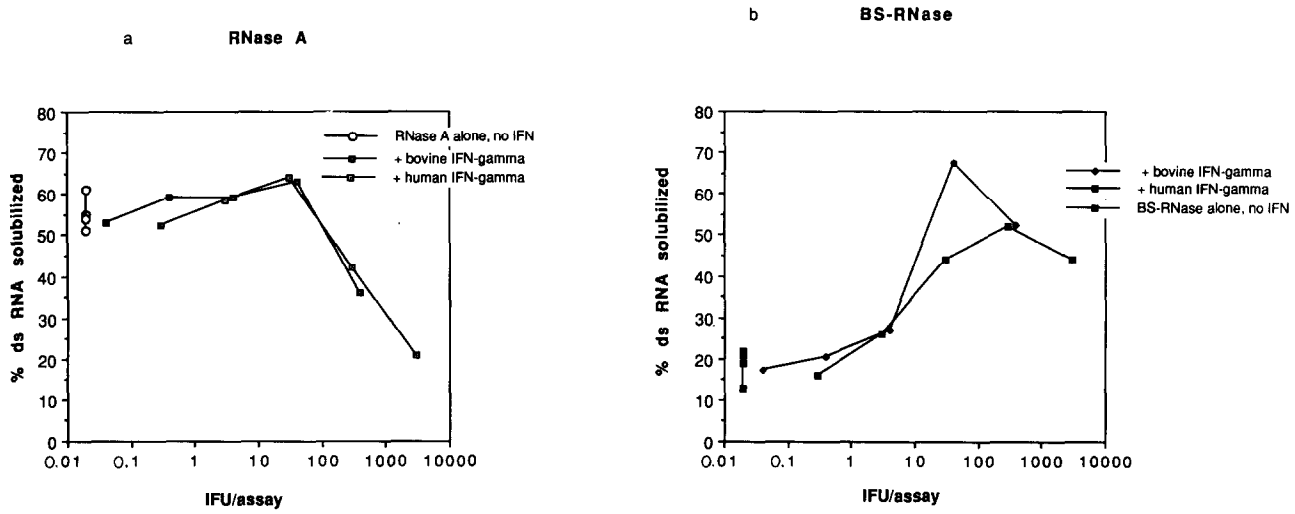

Fig. 1. Effect of human recombinant IFN-γΔCys and bovine IFN-γ on the activity of RNase A and bovine seminal RNase (BS-RNase) against ds-RNA. Complementary ss-RNA transcripts of a section of the T7 phage genome (ca. 300 bp long, cloned in opposite orientations downstream of the promoter for T7 RNA polymerase at the *Bam*HI site of plasmid pET-1 [12]) were prepared from *Eco*RV digests of the purified plasmids using T7-RNA polymerase. The ss-RNA was purified by polyacrylamide gel electrophoresis as previously described [8]. To make ds-RNA, equal amounts of the labeled ss-RNAs were combined, heated for 5 min to 55°C, and allowed to cool slowly to room temperature. The ds-RNA was degraded at least 100× more slowly by RNase A and had a much slower mobility on polyacrylamide gels than the corresponding ss-RNAs. (a) RNase A (0.02 ng/assay, diluted in 1 mg/ml Macaloid-treated BSA in 25 mM Tris-HCl, pH 7.9, (BSA/Tris)) was combined with dilutions of human and bovine IFN-γ in the same buffer. 'Nuclease assay mix' (ca. 200000 cpm/ml double-stranded ^{32}P-labeled RNA in 25 mM Tris/HCl, pH 7.9) was added (final sample volume was 8 μl), samples were incubated 40 min at 37°C, the assay was stopped with cold 10% TCA, and 5 μl of a solution of 20 mg/ml of BSA and 40 μg/ml of RNA were added as carriers. Samples were incubated for 30 min on ice, and centrifuged 10 min at 4°C in an Eppendorf centrifuge. The supernatant and pellet fractions were counted separately, and the data expressed as percent of total RNA in the supernatant fraction. (b) Ds-RNA degradation by BS-RNase (0.2 ng/assay) in the presence of varying concentrations of IFN-γ. Assays were essentially run as above but incubated 60 min at 37°C before TCA addition. Duplicate samples were averaged.

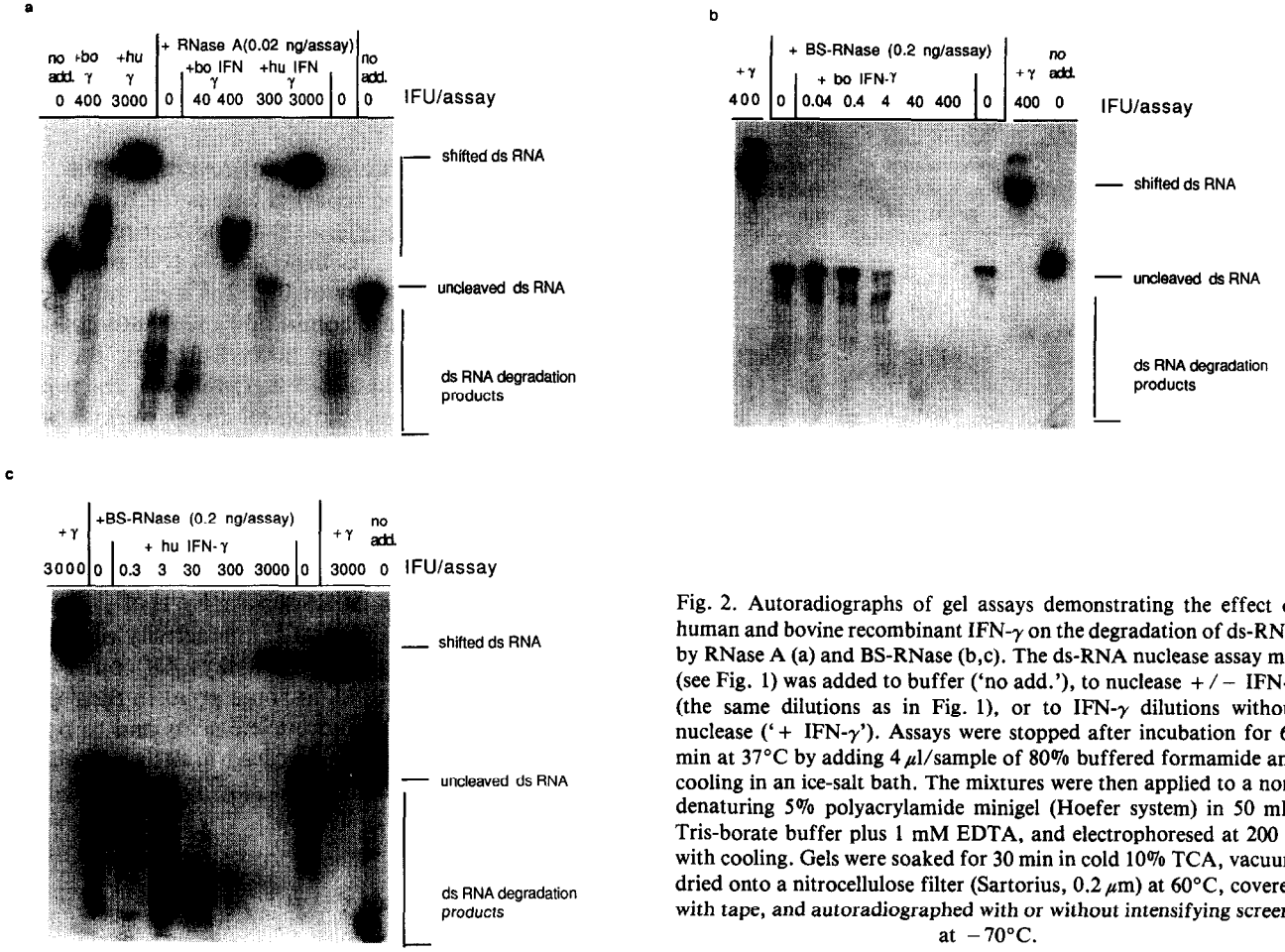

Fig. 2. Autoradiographs of gel assays demonstrating the effect of human and bovine recombinant IFN-γ on the degradation of ds-RNA by RNase A (a) and BS-RNase (b,c). The ds-RNA nuclease assay mix (see Fig. 1) was added to buffer ('no add.'), to nuclease + / − IFN-γ (the same dilutions as in Fig. 1), or to IFN-γ dilutions without nuclease ('+ IFN-γ'). Assays were stopped after incubation for 60 min at 37°C by adding 4 μl/sample of 80% buffered formamide and cooling in an ice-salt bath. The mixtures were then applied to a non-denaturing 5% polyacrylamide minigel (Hoefer system) in 50 mM Tris-borate buffer plus 1 mM EDTA, and electrophoresed at 200 V with cooling. Gels were soaked for 30 min in cold 10% TCA, vacuum dried onto a nitrocellulose filter (Sartorius, 0.2 μm) at 60°C, covered with tape, and autoradiographed with or without intensifying screens at −70°C.

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