A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon

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2-5A-dependent RNase is the terminal factor in the interferon-regulated 2-5A system thought to function in both the molecular mechanism of interferon action and in the general control of RNA stability. However, direct evidence for specific functions of 2-5A-dependent RNase has been generally lacking. Therefore, we developed a strategy to block the 2-5A system using a truncated form of 2-5A-dependent RNase which retains 2-5A binding activity while lacking RNase activity. When the truncated RNase was stably expressed at high levels in murine cells, it prevented specific rRNA cleavage in response to 2-5A transfection and the cells were unresponsive to the antiviral activity of interferon α/β for encephalomyocarditis virus. Remarkably, cells expressing the truncated RNase were also resistant to the antiproliferative activity of interferon. The truncated RNase is a dominant negative mutant that binds 2-5A and that may interfere with normal protein–protein interactions through nine ankyrin-like repeats.

Key words: 2-5A/ankyrin/interferon/ribonuclease/virus

Introduction

The interferon family of cytokines is responsible for a remarkably wide range of functions critical to higher organisms, including defense against viral infections, control of cell proliferation and differentiation, and modulation of the immune response (reviewed in Pestka et al., 1987). The development of an interferon response begins with the binding of interferon to specific cell surface receptors thus activating cascades of signal transduction reactions resulting in the transcription of distinct sets of genes (reviewed in Sen, 1991; Williams, 1991). The biological effects of interferons are mediated by the proteins encoded by the interferon-stimulated genes. The 2-5A system (Kerr and Brown, 1978) is an interferon-regulated, RNA degradation pathway which may also function in the general control of RNA stability in mammalian cells (Stark et al., 1979). Interferon treatment of cells induces at least four oligoadenylate synthetases and a unique RNase (reviewed in Sen and Lengyel, 1992). Double-stranded RNA (dsRNA) activates the synthetases resulting in the production of 5′-phosphorylated, 2′,5′-linked oligoadenylates known as 2-5A (Hovanessian et al., 1977; Kerr and Brown, 1978; Marie and Hovanessian, 1992). The only well-established function for 2-5A is its ability to activate the endoribonuclease, 2-5A-dependent RNase (Clemens and Williams, 1978; Zhou et al., 1993) cleaving RNA 3′ of UpUp and UpAp (Floyd-Smith et al., 1981; Wreschner et al., 1981b). However, the significance of this specificity with respect to the recognition of the natural RNA targets of 2-5A-dependent RNase remains unresolved.

There are several reasons why it has been difficult to establish specific functions for the 2-5A system. The main obstacle has been that there are basal levels of the synthetases and 2-5A-dependent RNase in most if not all mammalian cells. Although cell lines deficient in one or more of these enzymes have been described and partially characterized, the ‘missing’ enzyme can often be detected using sensitive methods (Krause et al., 1985a). Furthermore, in instances in which 2-5A-dependent RNase activity is undetectable, it is difficult to rule out transient and perhaps localized activation of 2-5A-dependent RNase (Nilsen and Baglioni, 1979). Despite the limitations inherent in the available methods and reagents, several studies have led to the conclusion that the 2-5A system functions in the antipicornavirus effects of interferon. For instance, elevated levels of 2-5A and specific rRNA cleavage products were correlated with inhibition of encephalomyocarditis virus (EMCV) replication in interferon-treated cells (Williams et al., 1979; Wreschner et al., 1981a; Silverman et al., 1982a). Furthermore, introduction of 2-5A into cells results in an inhibition of growth rates suggesting the 2-5A system may also be involved in the antiproliferative activity of interferon (Hovanessian and Wood, 1980). In addition, 2-5A-dependent RNase and synthetase levels were reported to be elevated in growth arrested or differentiated cells and reduced in rapidly dividing cells pointing to a potential broader role for the 2-5A system in the fundamental control of cell proliferation and differentiation (Stark et al., 1979; Jacobsen et al., 1983b; Krause et al., 1985b). In more direct evaluations of the role of the 2-5A system, constitutive expression of cDNAs encoding a low molecular weight form of 2-5A synthetase inhibited the replication of picornaviruses and reduced the cell growth rate (Chebath et al., 1987; Rysiecki et al., 1989; Coccia et al., 1990). However, because interferon induces both 2-5A-dependent RNase and multiple forms of 2-5A synthetase, increasing levels of just one form of synthetase may not completely mimic the effects of the 2-5A system obtained in interferon-treated cells.

The recent cloning of the murine and human 2-5A-dependent RNases provides important reagents with which to study the biological role of the 2-5A system (Zhou et al., 1993). Cloned human 2-5A-dependent RNase produced in vitro and naturally occurring 2-5A-dependent RNase have identical 2-5A binding properties and ribonuclease specificities (Zhou et al., 1993). Because 2-5A-dependent RNase mediates the effects of 2-5A in cells it is a logical target for blocking the 2-5A system. The requirement of 2-5A-dependent RNase for an activator suggested that a truncated form of the RNase might function as a dominant...
negative mutant. For example, expression of dominant negative mutants of the HIV rev protein (Malim et al., 1989), transforming growth factor β-1 (Lopez et al., 1992), epidermal growth factor receptor (Kashiles et al., 1991) and NFκB (Longeat et al., 1991), effectively blocked the activity of the wild type forms of these proteins in transfected cells. These mutants generally function by binding a required cofactor or substrate or by formation of heterodimers with the wild type protein, resulting in an inhibition of endogenous protein activity. Here we report that overexpression in murine SVT2 cells of a truncated form of murine 2-5A-dependent RNase, clone ZB1, which retains wild type 2-5A binding activity but lacks RNase function, does indeed result in an inhibition of the 2-5A system. The results implicate 2-5A-dependent RNase in the antiproliferative activity of interferon. Furthermore, nine ankyrin-like repeats were identified in the amino-terminal half of 2-5A-dependent RNase suggesting that the truncated RNase may interfere with normal protein—protein interaction (Lux et al., 1990; reviewed in Blank et al. (1992), and Nolan and Baltimore (1992)).

Results

2-5A-dependent RNase activity is inhibited by a truncated form of the RNase

To inhibit specifically the 2-5A system, we investigated whether a truncated form of murine 2-5A-dependent RNase could function as a dominant negative mutant. The truncated, murine 2-5A-dependent RNase, encoded by clone ZB1, lacks a carboxy-terminal region consisting of 89 amino acid residues but it nevertheless has 2-5A binding activity which is indistinguishable from the full-length enzyme (Zhou et al., 1993). Translation reactions were performed in rabbit reticulocyte lysate depleted of endogenous 2-5A-dependent RNase with an affinity matrix, 2-5A—cellulose (Materials and methods; Silverman, 1985; Zhou et al., 1993). Synthesis of comparable levels of the wild type and truncated RNases were monitored with [35S]methionine (Figure 1A, lanes 1 and 2). RNase assays were performed by isolating unlabeled translation products on the activating affinity matrix, 2-5A—cellulose, followed by washing to remove general nucleases and then by incubating with radiolabeled poly(U) as substrate (Silverman, 1985). Previous studies showed that 2-5A-dependent RNase from murine L cells or recombinant human 2-5A-dependent RNase produced in vitro were active in this assay (Silverman, 1985; Zhou et al., 1993). In contrast, no RNase activity was detected in reactions containing the truncated murine 2-5A-dependent RNase clone ZB1 (data not shown and Figure 1B). To determine whether the truncated RNase could inhibit the wild type protein in vitro, ribonuclease assays were performed with a constant amount of the complete, human 2-5A-dependent RNase in the absence or presence of increasing levels of the truncated RNase, ZB1. Interestingly, the human 2-5A-dependent RNase was strongly inhibited by addition of the truncated RNase (Figure 1B). The results indicated that clone ZB1 had the potential to function as a dominant negative mutant in cells.

Stable, high level expression of truncated 2-5A-dependent RNase in a murine cell line

To extend these studies to intact cells, clone ZB1 cDNA driven by the SV40 late promoter in plasmid pSVL was stably expressed in interferon-responsive murine SVT2 cells (Materials and methods). Levels of mutant (clone ZB1) and endogenous 2-5A-dependent RNases were measured after covalent cross-linking to a 32P-labeled and bromine-substituted 2-5A analog under ultraviolet light (Figure 2; Nolan-Sorden et al., 1990). 2-5A binding activity, a measure of 2-5A-dependent RNase levels, was 25-fold lower in SVT2/pSVL cells (containing vector lacking cDNA) than in a cell line expressing clone ZB1 cDNA (SVT2/ZB1 cells) (as determined by PhosphorImager analysis of dried gels, Molecular Dynamics). Because protein encoded by clone ZB1 is a truncated form of 2-5A-dependent RNase it migrated slightly faster in the gel (Figure 2, lanes 4–8) relative to endogenous 2-5A-dependent RNase (lanes 1–3). The relative overexpression of the truncated RNase suggested that activation of the endogenous 2-5A-dependent RNase by 2-5A might be compromised in the SVT2/ZB1 cells.
Fig. 3. 2-5A-mediated rRNA cleavage is inhibited in cells expressing high levels of the truncated 2-5A-dependent RNase. Control, SVT2/pSVL cells (lanes 1–3) or SVT2/ZB1 cells (lanes 4–6) were incubated without (lanes 1, 2, and 5) or with 1000 units/ml of interferon (α + β) for 16 h (lanes 3 and 6) and were then (lanes 2, 3, 5 and 6) transfected with 5 μM 2-5A. Total cellular RNA was isolated and 15 μg RNA per lane were electrophoresed on a glyoxal–agarose gel (Wreschner et al., 1981a). An autoradiogram of a Northern blot probed with radiolabeled cDNA to human 18S rRNA is shown. Positions of 28S and 18S rRNA and the 2-5A-mediated 18S rRNA breakdown products are indicated.

The truncated RNase inhibits endogenous 2-5A-dependent RNase activity in intact cells

2-5A-dependent RNase cleaves rRNA in intact ribosomes resulting in a characteristic set of discrete RNA cleavage products (Wreschner et al., 1981a; Silverman et al., 1983). For instance, 2-5A-mediated rRNA cleavages were previously observed in interferon-treated, EMCV-infected cells (Wreschner et al., 1981a; Silverman et al., 1983). Therefore, to determine the effect of the truncated RNase on endogenous 2-5A-dependent RNase activity in intact cells, 2-5A-mediated cleavage of 18S rRNA was determined in Northern blots probed with radiolabeled cDNA for 18S rRNA. In the control cells, specific 18S rRNA cleavage products were clearly observed after transfecting cells with 2-5A by calcium phosphate coprecipitation (Figure 3, lane 2). Pretreatment of the control cells with interferon followed by 2-5A transfection resulted in a significant enhancement in the specific breakdown of 18S rRNA due to the interferon induction of 2-5A-dependent RNase (Figure 3, lane 3). In contrast, in the SVT2/ZB1 cells, the ability of 2-5A to induce degradation of the 18S rRNA was severely impaired, regardless of whether or not the cells were pretreated with interferon (Figure 3, lanes 4–6). For comparison, another clonal cell line, SVT2/ZB1.4, which expressed the truncated RNase at only 1.2–2-fold higher levels than the endogenous enzyme, showed no reduction in 18S rRNA cleavage after 2-5A transfection of the interferon treated cells. These findings suggest that relatively high levels of the truncated RNase are required to detect inhibition of endogenous 2-5A-dependent RNase by this assay (data not shown).

Fig. 4. Interferon induced PKR activity to the same extent in control, SVT2/pSVL and in SVT2/ZB1 cells. PKR from control, SVT2/pSVL (lanes 1 and 2) and SVT2/ZB1 (lanes 3 and 4) incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1000 units/ml of interferon (α + β) for 16 h was adsorbed to the activating affinity matrix, poly(I)−poly(C)−cellulose (Materials and methods). Autophosphorylation of PKR was determined with [γ−32P]ATP as substrate. The positions of the molecular weight markers (in kDa) and PKR are indicated in the autoradiogram of the dried SDS−polyacrylamide gel.

Interferon induction of PKR is not impaired in the SVT2/ZB1 cells

Although the 2-5A system and the dsRNA-dependent protein kinase, PKR, perform very different biochemical functions there is some overlap with respect to the effects of the two pathways in interferon treated cells. In particular, both the 2-5A system and PKR have been implicated in the antipicornavirus activity of interferon (Chebath et al., 1987; Meurs et al., 1992). In this regard, the dominant negative mutant, clone ZB1, is expected to be a specific inhibitor of the 2-5A system. However, to rule out effects of clone ZB1 on the PKR pathway, we monitored induction of PKR by interferon. PKR activity was assayed after isolation and purification of the kinase on the activating affinity matrix, poly(I)−poly(C)−cellulose (Wells et al., 1984; Silverman and Krause, 1987). Levels of autophosphorylation with [γ−32P]ATP as substrate were determined in autoradiograms after gel electrophoresis. A similar induction of PKR activity by interferon was obtained with both SVT2/pSVL and SVT2/ZB1 cells (Figure 4). Therefore, expression of the mutant RNase does not affect PKR activity as determined by this assay. The possible inhibition of PKR in the intact cells, however, is not completely excluded by these data. These results further indicate that the transfected cells were not generally defective in interferon stimulation of gene expression.

The SVT2/ZB1 cells are resistant to the anti-EMCV activity of interferon

To determine whether the truncated RNase could block a recognized biological effect of the 2-5A system, EMCV replication was measured in interferon-treated SVT2/ZB1 and SVT2/pSVL cells. For comparison, VSV replication was determined owing to its reported resistance to the 2-5A system (Chebath et al., 1987; Rysiecki et al., 1989; Coccia et al., 1990). Cells were first preincubated in the absence
or presence of murine interferon ($\alpha + \beta$) and then were infected with EMCV or VSV (Materials and methods). Interferon treatment of the SVT2/pSVL cells resulted in a dose-dependent reduction in EMCV yield (Figure 5A). Indeed, 10 units/ml of interferon reduced viral replication by 200-fold and 1000 units/ml of interferon produced a 4000-fold reduction in EMCV yield (Figure 5A). In contrast, in the SVT2/ZBI cells, interferon concentrations as high as 100 units/ml did not significantly inhibit EMCV replication (Figure 5A). Furthermore, 1000 units/ml of interferon produced only a 16-fold reduction in virus yield in the SVT2/ZBI cells providing strong supportive evidence that the 2-5A system functions in the anti-EMCV activity of interferon. Relatively high levels of the truncated RNase were required for this effect because the SVT2/ZBI1.4 cells, which expressed only low levels of the truncated RNase, retained sensitivity to the anti-EMCV effect of interferon (data not shown).

In contrast, interferon protection from VSV challenge was similar in the SVT2/ZBI and control cell lines (Figure 5B). There was only a 2.5-fold reduction in interferon protection against VSV in the SVT2/ZBI1 cells as compared with the control cells (at 1000 units/ml of interferon there was 5.6 and 6.0 log$_{10}$ units of reduction in VSV yield, respectively). These results indicate that while the 2-5A system is a potent inhibitor of EMCV replication, the anti-VSV effect of interferon is predominantly mediated by another unidentified pathway.

The truncated 2-5A-dependent RNase suppresses the antiproliferative activity of interferon

To determine the involvement of 2-5A-dependent RNase in the antiproliferative activity of interferon, growth rates of SVT2/pSVL and SVT2/ZBI cells were compared in the presence or absence of interferon ($\alpha + \beta$) (Figure 6). The control, SVT2/pSVL cells were relatively sensitive to the growth inhibitory activity of interferon. Indeed, in four separate experiments interferon treatment of the control cells resulted in a 53–98% reduction in the cell growth rates as determined by measuring cell population numbers as a function of time (Figure 6A; data not shown). After several days of interferon treatment, cell division apparently ceased for the SVT2/pSVL culture (Figure 6A). In contrast, interferon treatment of the SVT2/ZBI1 cells resulted in only a minimal (0–33%) often transient reduction in the growth rate (Figure 6B; data not shown). Within each experiment, the antiproliferative effect of interferon on control cells was always at least twice that seen in cells expressing the truncated RNase. The resistance of the SVT2/ZBI1 cells to the antiproliferative activity of interferon was also observed by measuring $[^{3}H]$thymidine incorporation into DNA on the last day of these experiments. Interferon treatment of the control cells caused a 44–50% reduction in $[^{3}H]$thymidine incorporation while there was only a 0–10% reduction in $[^{3}H]$thymidine incorporation in the SVT2/ZBI1 cells (data not shown). To demonstrate that the interferon-resistant phenotype observed in the SVT2/ZBI1 cells was not limited to one clonal cell line, the SVT2/ZBI1.4 cells were cultured in the presence and absence of interferon. Accordingly, the antiproliferative activity of interferon was suppressed to the same extent in both clonal cell lines expressing the truncated RNase (compare Figures 6B and C; see Discussion).

Discussion

Expression of dominant negative mutants is a method for determining the functions of proteins in intact cells (e.g. Malim et al., 1989; Longeget et al., 1991). Here we demonstrate that the 2-5A system was effectively blocked by expressing high levels of a truncated 2-5A-dependent RNase having 2-5A binding activity but lacking RNase function. Although we have determined that the carboxy-terminus of 2-5A-dependent RNase is required for ribonuclease activity (Figure 1B), the precise localization of the ribonuclease domain remains to be determined.

The 2-5A system inhibits EMCV replication in interferon treated cells

An impressive reduction in the interferon inhibition of EMCV replication was obtained in the SVT2/ZBI1 cells (Figure 5A). The results provide a quantitative measure of
the extent to which the 2-5A system inhibits EMCV replication thus confirming and extending several studies linking the 2-5A system to the antipicornavirus activity of interferon (Williams et al., 1979; Wreschner et al., 1981a; Silverman et al., 1982a; Watling et al., 1985; Chebath et al., 1987; Kumar et al., 1988; Rysiecki et al., 1989; Coccia et al., 1990; Gribaudo et al., 1991). For instance, overexpression of cDNA for the 40 kDa form of 2-5A synthetase produced up to an ~1000-fold reduction in mengo virus yield (Chebath et al., 1987). Another study demonstrated that a 2-5A analog inhibited interferon protection against EMCV, but only by <10-fold (Watling et al., 1985). Apparently, expression of the dominant negative RNase mutant results in a much more efficient inhibition of endogenous 2-5A-dependent RNase than does transfection with the 2-5A analog. Nevertheless, when considered together, results presented in this study and in previous reports provide compelling evidence that the 2-5A system is an important mediator of the antipicornavirus activity of interferon.

Interestingly, the SVT2/ZBI cells were resistant to the anti-EMCV effect of interferon despite a strong interferon induction of PKR, also implicated in the molecular mechanism of EMCV inhibition (Figure 4; Meurs et al., 1992). PKR activation, therefore, is the likely cause for the modest inhibition of EMCV in the SVT2/ZBI cells observed at 1000 units/ml of interferon (Figure 5A). Perhaps both PKR and the 2-5A system are necessary to obtain an effective antipicornavirus response to interferon.

The rhabdovirus, VSV, is very sensitive to inhibition by interferon (Figure 5B). Nevertheless, expression of the dominant negative RNase mutant reduced the inhibition in VSV yield at 1000 units/ml of interferon by only ~2.5-fold (Figure 5B). Similarly, constitutive expression of 2-5A synthetase resulted in protection from EMCV and mengo virus, but not from VSV (Chebath et al., 1987; Rysiecki et al., 1989; Coccia et al., 1990). It is apparent from this study that interferon is a potent inhibitor of VSV replication by mechanisms which are largely independent of the 2-5A system. These findings support the hypothesis that there are multiple antiviral mechanisms of interferon action. In more general terms, these results also demonstrate that cellular expression of the dominant negative RNase mutant followed by virus infection will determine which types of viruses are sensitive to inhibition by the 2-5A system.

The role of the 2-5A system in the antiproliferative activity of interferons

The resistance of cells expressing the truncated RNase to the anticalcellular activity of interferon suggests that 2-5A-dependent RNase mediates growth suppressor effects of interferon. Similarly, constitutive expression of 2-5A synthetase produced a reduction in cellular growth rates (Rysiecki et al., 1989). Additional support for this hypothesis is provided by the occurrence of 2-5A oligonucleotides in interferon treated murine L cells (Knight et al., 1980) and by the observation that 2-5A transfection inhibits cell growth rates (Hovanessian and Wood, 1980). Although in one study no 2-5A (<1 nM) was found in interferon-treated human Daudi cells, the possibility of transient or localized production of 2-5A was not completely excluded (Silverman et al., 1982b).

The interferon-resistant phenotype of the SVT2/ZBI cells was obtained despite normal expression of PKR (Figure 4). Previous studies showed that expression of PKR cDNA produced a slow-growth phenotype in yeast cells, which contain dsRNA activators of PKR, but not in murine NIH 3T3 cells (Chong et al., 1992; Meurs et al., 1992). Nevertheless, mutant forms of PKR produced tumors in nude mice, suggesting a tumor suppressor function for PKR (Koromilas et al., 1992; Meurs et al., 1993). The potential contribution of PKR to the interferon-induced growth suppression in SVT2/pSVL cells appears to be marginal because there were similar levels of PKR activity in the interferon-resistant SVT2/ZBI cells. However, perhaps both the 2-5A system and PKR need to be functional to produce a significant antiproliferative response to interferon treatment. Alternatively, the type of cell being studied will not doubt affect the extent to which each pathway contributes to interferon-mediated growth suppression.

Interestingly, although the SVT2/ZBI.1.4 cells express only 1.2–2 times as much truncated RNase as wild type RNase, they are nevertheless resistant to the antignovirus activity of interferon (Figure 6C). The low levels of the truncated RNase in these cells were insufficient to block either the breakdown of 18S rRNA after transfection of the cells with 2-5A or the anti-EMCV effect of interferon. However, in vitro assays indicated that 2-5A-dependent RNase is very sensitive to inhibition by the truncated form of the protein (Figure 1). Therefore, the rRNA cleavage and EMCV assays may lack the sensitivity required to detect the effects of low levels of the truncated RNase. Alternatively, suppression of the antiproliferative and anti-EMCV effects of interferon may involve different functions of the truncated RNase (see next section).

The growth suppressing effect of interferon may involve induction of both 2-5A-dependent RNase (Jacobsen et al., 1983a) and 2-5A synthetases (Hovanessian et al., 1977). However, the source of the dsRNA activators of the synthetases and the RNA targets of the 2-5A-dependent RNase remain to be determined. In fact, the fundamental question of whether the 2-5A pathway activity results in a global or targeted ablation of RNA is unknown but will be addressed with cells expressing the dominant negative 2-5A-dependent RNase mutant.

The truncated RNase may interfere with protein–protein interactions mediated by ankyrin repeats

Although it is likely that the truncated RNase functions as a dominant negative mutant at least in part through its 2-5A binding activity, additional mechanisms of action are not ruled out by the data. In this regard, computer-assisted comparison (Altschul et al., 1990) of the murine and human 2-5A-dependent RNases to sequences present in several data bases identified the presence of nine ankyrin-like repeats (the last repeat is incomplete) (Figure 7; Breeden and Nasmyth, 1987; Lux et al., 1990). Ankyrin repeats are implicated in mediating intra- and intermolecular protein–protein interactions in many different types of proteins. For instance these repeated elements are believed to function in human erythrocyte ankyrin by binding with membrane proteins and tubulin (Lux et al., 1990), in IxB by binding to transcription factor NFκB, and within the NFκB precursor, p105 [reviewed in Blank et al. (1992), and Nolan and Baltimore (1992)] and in transcription factor subunits GABPβ with
GABPα (Thompson et al., 1991). The relative positions of the ankyrin repeats in 2-5A-dependent RNase to the 2-5A binding domain, the cysteine-rich region, a protein kinase homology region, and the carboxy-terminal region required for RNase activity are shown (Figure 7B; Zhou et al., 1993). The region containing the ankyrin repeats is intact in the truncated RNase (clone ZB1) which lacks sequence from the carboxy-terminus. Therefore, it is likely that the truncated RNase disrupts natural protein—protein interactions. Although the effects of the truncated RNase could be explained by the formation of inactive heterodimers with the wild type RNase, our recent findings suggest that this is unlikely. Recombinant human 2-5A-dependent RNase produced in insect cells from a baculovirus vector was purified by FPLC (Pharmacia). The recombinant RNase eluted from a gel filtration column as a monomer and it showed potent and highly specific, 2-5A-dependent RNase activity (B.Dong, L.Xu, X.Lee and R.H.Silverman, unpublished).

The ankyrin repeats are probably essential to the normal, physiological functioning of 2-5A-dependent RNase. In this regard, the two phosphate binding loop (P-loop) motifs (Walker et al., 1982; Saraste et al., 1990), glycine-lysine-threonine, involved in 2-5A binding activity (Zhou et al., 1993) occupy identical positions in the seventh and eighth ankyrin repeats (Figure 7A). Perhaps, in the inactive form of the enzyme, there is an interaction between the ankyrin-repeat region, consisting of the amino-terminal half of the protein, with the carboxy-terminal half of 2-5A-dependent RNase. Binding of 2-5A could induce a conformational change in the enzyme causing the disassociation of these two regions thereby releasing the catalytic function of the RNase. Furthermore, the presence of the ankyrin repeats raises the intriguing possibility that the enzyme may have functions other than its RNase activity. For instance, like the IxBs the 2-5A-dependent RNase could regulate the activity of another protein. Perhaps such interactions are controlled by the binding of 2-5A to the 2-5A-dependent RNase. For example, 2-5A binding could affect the affinity of the RNase for a particular subcellular compartment by changing protein—protein contacts. Finally, it is interesting to note that many of the proteins that contain ankyrin repeats function in differentiation and cell cycle control (Breeden and Nasmyth, 1987; Lux et al., 1990). Similarly, the 2-5A-dependent RNase is induced during differentiation of murine embryonal carcinoma cells (Krause et al., 1985b) and during growth arrest by confluency (Jacobsen et al., 1983b). Interestingly, 2-5A-dependent RNase is the only nuclease
we could identify which contains ankny repeats.

The expression of the 2-5A system is tightly correlated with cellular growth rates, hormone-status and state of cell differentiation (Stark et al., 1979; Jacobsen et al., 1983b; Krause et al., 1985b). Indeed, the widespread occurrence of the 2-5A pathway in reptiles, avians and mammals suggests an important function for the 2-5A system in the general control of RNA decay (Cayley et al., 1982). Results presented in this study suggest that 2-5A-dependent RNase is an important mediator of the antiviral and anticytotoxic activities of interferon. Therefore, the truncated 2-5A dependent RNase is a valuable reagent for determining the involvement of the 2-5A system in interferon action and in the control of RNA decay during cell growth and differentiation.

Materials and methods
Synthesis of mRNA and protein in vitro.
T3 or T7 phage polymerase was used to transcribe plasmid pZBI encoding the truncated form of murine 2-5A-dependent RNase and plasmid pZCS encoding the full-length human 2-5A-dependent RNases (Zhou et al., 1993) in the presence of m7GppG as described (Promega) except the transcription reactions were supplemented with 15% dimethyl sulfoxide and incubations were at 37°C for 60 minutes. RNA was purified by centrifugation through Sephadex G50 and then ethanol-precipitated. Protein synthesis was as described (Promega) in the presence of 50 μM zinc sulfate at 30°C for 1 hour in rabbit reticulocyte lysate pretreated with micrococcal nuclease. The endogenous, rabbit 2-5A-dependent RNase was removed prior to translation by absorption to 2-5A [poly(A2’p)5-A-cellulose prepared as described by Wells et al., 1984], and Silverman and Krause (1987) for 1 hour on ice (Zhou et al., 1993). Translation reactions in the deected lysates were with the mRNA for truncated murine RNase (ZB1) or the full-length human RNase (ZCS).

Assay of 2-5A-dependent RNase activity
The 2-5A-dependent RNase assay was performed as described by Silverman (1985). Briefly, proteins produced in the reticulocyte translation system were adsorbed to 30 μg/ml 2-5A-cellulose for 2 hours on ice. The matrix was then washed by centrifuging and resuspending three times in buffer A (Silverman, 1985). RNase activity was determined by incubation with poly(U)[32P]Cp, 16 μM in nucleotide equivalents at 37°C for 2 hours. The levels of acid-precipitable, radioactive RNA were determined by filtration on glass fiber filters (Silverman, 1985).

Mammalian cell culture
Murine SVT2 cells, an SV40 T antigen expressing 3T3 cell line and clonal cell lines were grown in Eagle's Modified Medium (EMEM, Biowhittaker) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% balanced air.

Expression vectors and transfections
The murine ZB1 cDNA, encoding the truncated form of 2-5A-dependent RNase, was directionally subcloned into the pSec1 (5') and BamHI (3') sites in plasmid pSVL (Pharmacia) expression vector by standard methods (Sambrook et al., 1989).

The amino acid sequence of truncated, murine 2-5A-dependent RNase was reported previously (Zhou et al., 1993). Clone ZB1 encodes amino acid residues 1-656 of murine 2-5A-dependent RNase, except an apparent cloning artifact altered the last five amino acid residues from NPQGD to KPLSG. Plasmid pSVL or construct plasmid pSVL/ZB1(10 μg each) were cotransfected with 1 μg pcDNANeo (Invitrogen) by calcium phosphate coprecipitation (Sambrook et al., 1989). Stable transfectants were selected by culturing in the presence of 475 μg/ml G418 (Gibco BRL) and individual clones were isolated for analysis of expression.

Detection of 2-5A-dependent RNase with a 2-5A probe
The synthesis of the 2-5A probe, pA2[pA(32P)]CpA2[pA]Cp and its crosslinking to the RNase were performed exactly as described by Nolan-Sorden et al. (1990) with 2-5A analog which was generously provided by Dr. Paul F. Torrence (Bethesda). Briefly, 0.7-2.5 mM (3000 Ci/mmol) of 2-5A probe was incubated for 1 hour on ice with cell extract prepared as described by Nolan-Sorden et al. (1990). Covalent cross-linking was done under an ultraviolet lamp (308 nm) for 1 hour on ice and the proteins were separated on SDS-10% polyacrylamide gels.

2-5A transfections and analysis of rRNA cleavage
SVT2/pSVL or SVT2/ZB1 cells were seeded at 5 × 10⁴ cells per 100 mm plate and treated with 1000 units/ml interferon (α + β) for 18 hours as indicated. Cells were then transfected with 5 μg 2-5A trimer triphosphate by calcium phosphate coprecipitation (Sambrook et al., 1989). Incubation of 2-5A with the cells was for 75 minutes after which cells were washed, refed and incubated for an additional 3.5 hours before harvesting. Total RNA was isolated using the RNAzol reagent (TelTest; Chomczynski and Sacchi, 1987). RNA samples were electrophoresed on a glyoxal-agarose gel (Wreschner et al., 1981a) and transferred to Nytran membrane (Scherleicher and Schuell). The RNA in the blot was hybridized to a 32P-labeled human 18S rRNA cDNA probe (kindly provided by Dr. John Thaden, Baltimore).

Assay of PKR activity
PKR activity was assayed by autophosphorylation using the activating affinity matrix, poly(I)·poly(C)−cellulose, as described by Wells et al. (1984) and Silverman and Krause (1987) with the following modifications. Cell extracts (50 μg of protein per assay) were incubated with 25 μg per ml of poly(I)·poly(C)−cellulose for 1 hour and the matrix was washed three times and incubated in the presence of [γ-32P]ATP (2 μCi per assay; 15 Ci/mmol), 20 mM 1,000 HEPES (pH 7.5), 80 mM potassium chloride, 1 mM magnesium chloride, 1 mM manganese chloride and 0.1 mM 2-mercaptoethanol in a final volume of 20 μl for 30 minutes at 30°C. The proteins were separated on a 10% polyacrylamide gel and analyzed by autoradiography.

Measuring the antiviral activity of interferons
SVT2/pSVL or SVT2/ZB1 cells were seeded in 24-well plates (Costar) at a density of 1 × 10⁴ cells per well. After 24 hours of seeding, cells were treated with various dilutions of interferon for 18 hours. Cells were then infected with VSV or EMVC at an m.o.i. of 1.0 in EMEM containing 2% FBS for 1 hour. Medium was removed and the cells were washed with phosphate buffered saline, then refed with EMEM containing 10% FBS and incubated for 15 hours. The medium containing progeny virions was harvested and diluted for titration. Inducer SVT2 cells were seeded in 24-well plates at 5 × 10⁴ cells per well and at 24 hours post-seeding, cells were infected with virus dilutions, in duplicate, and incubated for an additional 16 hours. Viable cells were stained with neutral red and destained to quantify cell killing as described by Finter (1981).

Measuring cell proliferation
For growth experiments, cells were seeded at 550 cells/cm² in 12-well plates and culture medium was changed every other day. Cells were treated with interferon (α + β) (1.8 × 10⁶ units/mg protein) (Lee Biomolecular) as described (see text). Cell numbers were determined from duplicate or triplicate cultures using a hemocytometer.

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