Antagonism of 2–5 A-mediated inhibition of protein synthesis in intact cells by 2′,5′-$(pA)_3$

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In vitro studies have shown that the translational inhibitory activity of 2–5 A can be blocked by the oligoribonucleotide 2′,5′-$(pA)_3$. We have examined the effect of simultaneous introduction of inhibitor and antagonist into intact mouse cells using calcium phosphate coprecipitation. Upon introduction of 10^{-4} M 2′,5′-$(pA)_3$ and 10^{-6} M 2–5 A, inhibition of protein synthesis was prevented. Efficiency of calcium phosphate precipitation of 2–5 A in the presence or absence of 2′,5′-$(pA)_3$ was comparable. Introduction of 2′,5′-$(pA)_3$ analogs showed that nucleotides which do not bind well to the 2–5 A dependent endonuclease do not prevent 2–5 A inhibitory activity. Thus, 2′,5′-$(pA)_3$ functions as an antagonist of 2–5 A in vivo.

Interferon *2–5 A* *Protein synthesis*

1. INTRODUCTION

Two interferon-induced enzymatic pathways leading to inhibition of translation have been identified [1,2]. First, the protein kinase pathway involves phosphorylation of the small subunit of eukaryotic initiation factor, eIF-2, resulting in inhibition of protein synthesis initiation. Second, the 2–5 A pathway involves induction of a 2–5 A synthetase which, in the presence of ATP and double-stranded RNA, synthesizes 2–5 A, an activator of a latent endonuclease which degrades mRNA. The role of these pathways in the antiviral effect of interferon is unclear and may depend on characteristics of specific virus-cell interaction [3–9].

To evaluate the roles of the protein kinase and 2–5 A pathways, we have developed a method by which the 2 enzyme systems might be functionally distinguished by specific antagonism of the 2–5 A pathway using a synthetic oligoribonucleotide. It has been previously shown that inhibition of protein synthesis by exogenously added 2–5 A could be prevented by the oligoribonucleotide, 2′,5′-$(pA)_3$, in in vitro studies in mouse L cell-free systems [10,11]. In the present experiments, oligonucleotides have been introduced into intact mouse L cells using a modification of the calcium phosphate coprecipitation technique of Graham and Van der Eb [12]. Under these conditions, we have found that 2′,5′-$(pA)_3$ when introduced into cells, is able to stimulate protein synthesis and, when introduced together with 2–5 A, is able to prevent the protein synthesis inhibiting action of 2–5 A. Recently, Watling et al. [13] reported the use of an analog of 2–5 A to accomplish this purpose and to

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Abbreviations: 2′,5′-$(pA)_3$, p5′A2′p5′A2′p5′A; 3′,5′-$(p2'dA)_3$, p5′(2′dA)3′p5′(2′dA)3′p5′(2′dA)

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prevent the anti-encephalomyocarditis virus action of interferon.

2. MATERIALS AND METHODS

Mouse L cells were maintained in Dulbecco's modified Eagles minimum essential medium (MEM) containing 10% heat-inactivated fetal calf serum.

The calcium phosphate coprecipitation technique used for transfection was a modification of the method of Panet et al. [14]. To assess the number of cells present following the transfection, cells were washed with leucine-free MEM and incubated at 37°C in leucine-free MEM containing 10 μCi/ml L-[4,5-^{3}H(n)]leucine. After 1 h, the medium was aspirated and the procedure in [14] followed.

The oligonucleotides employed in this study were prepared either as described in [15,16] for 2-5 A trimer and 2′,5′-(pA)$_3$ or were obtained from commercial sources: poly(A) and 3′,5′-(p2′dA)$_3$ from P.-L. Biochemicals (Milwaukee, WI) and 5′-AMP from Aldrich (Milwaukee, WI). The [^{3}H]2-5 A was prepared from [^{3}H]ATP (Amersham-Searle, Chicago, IL; final spec. act. 45 × 10^6 cpm/μmol) by incubation with 2-5 A synthetase from interferon-treated mouse L cells adsorbed to poly(I)-poly(C)-Sepharose, according to [17]. The 2-5 A trimer 5′-triphosphate was purified by high-performance liquid chromatography on a Bondapack column with a 0-20% gradient of ammonium phosphate (50 μM, pH 7) into methanol/water (1:1). The product had a final specific activity of 1.4 × 10^6 cpm/A_{260} unit.

3. RESULTS

The results from a single transfection experiment in which 2-5 A and 2′,5′-(pA)$_3$ were introduced simultaneously into intact cells are presented in fig.1. The extent to which protein synthesis was inhibited by 2-5 A was dependent upon the concentration of the oligonucleotide. At 10^{-9} M, 2-5 A did not have any effect on protein synthesis. However, as the concentration of 2-5 A approached 10^{-6} M, the level of protein synthesis dropped to less than 30% of the control. In contrast, protein synthesis in cells exposed to precipitates containing only 2′,5′-(pA)$_3$ was significantly

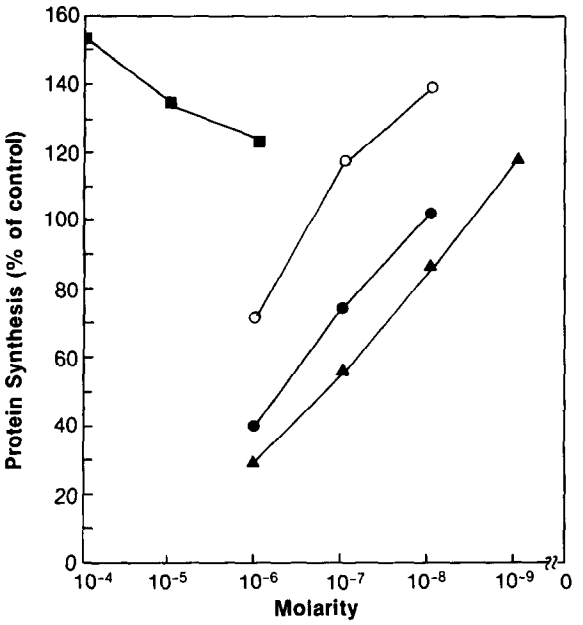

Fig.1. Effect of 2′,5′-(pA)$_3$ on inhibition of protein synthesis by 2-5 A. Mouse L cells, labeled prior to transfection with 10 μCi/ml [^{3}H]leucine, were exposed to varying concentrations of calcium phosphate-precipitated 2′,5′-(pA)$_3$ (■), 2-5 A (▲), 10^{-4} M 2′,5′-(pA)$_3$ and 2-5 A at the indicated concentrations (○), 10^{-5} M 2′,5′-(pA)$_3$ and 2-5 A at the indicated concentrations (●). Each determination represents an average of 4 replicate samples. The level of 100% protein synthesis is defined as cpm [^{35}S]Met/cpm [^{3}H]Leu as determined in mock-transfected cells.

enhanced and this stimulation was dose-dependent.

When 2-5 A and 2′,5′-(pA)$_3$ were simultaneously introduced into L cells, inhibition of protein synthesis by 2-5 A was effectively reduced and this reduction was dependent upon the concentration of 2′,5′-(pA)$_3$. For example, introduction of 10^{-6} M 2-5 A into the cells resulted in a reduction in the level of protein synthesis to 29%. When 2′,5′-(pA)$_3$ was present at 10^{-5} M, the level of protein synthesis was reduced by 2-5 A to only 40%. Furthermore, when 2′,5′-(pA)$_3$ was present at 10^{-4} M, the level of protein synthesis was only slightly reduced to 72%. A similar antagonistic effect of 2′,5′-(pA)$_3$ on inhibition of protein synthesis by 2-5 A at 10^{-7} and 10^{-8} M was also observed. However, only when less effective concentrations of 2-5 A (10^{-7}, 10^{-8} M) were introduced did

2′,5′-$(pA)_3$ overcome the inhibitory properties of the 2–5 A and bring levels of protein synthesis up to those of mock-transfected control cells.

The specificity of antagonism was investigated by examining the effect of several 2′,5′-$(pA)_3$ related adenosine-containing nucleotides, each simultaneously transfected with 2–5 A. The selected compounds had been evaluated previously in an L cell-free protein synthesis system and were observed (i) to have no effect on inhibition of protein synthesis by 2–5 A and (ii) to lack any inhibitory properties of their own (not shown; see [18] for reference). The results from several transfection experiments in which 2′,5′-$(pA)_3$ and various adenosine nucleotides were compared for the ability to antagonize 2–5 A-mediated inhibition of protein synthesis are summarized in fig.2. The level of protein synthesis inhibition in the presence of 2–5 A is designated as 100%; the level of protein synthesis observed in the presence or absence of other nucleotides is expressed as a percentage relative to that value. The cointroduction of 2′,5′-$(pA)_3$, at a concentration, based on adenosine residues (M_A), of 3×10^{-4} M_A with 10^{-7} M 2–5 A effectively increased the level of protein synthesis from 100 to 136% (fig.2B). In contrast, the simultaneous introduction of 3′,5′-(p2′dA)$_3$ (3×10^{-4} M_A) with 2–5 A (10^{-7} M) did not significantly affect the level

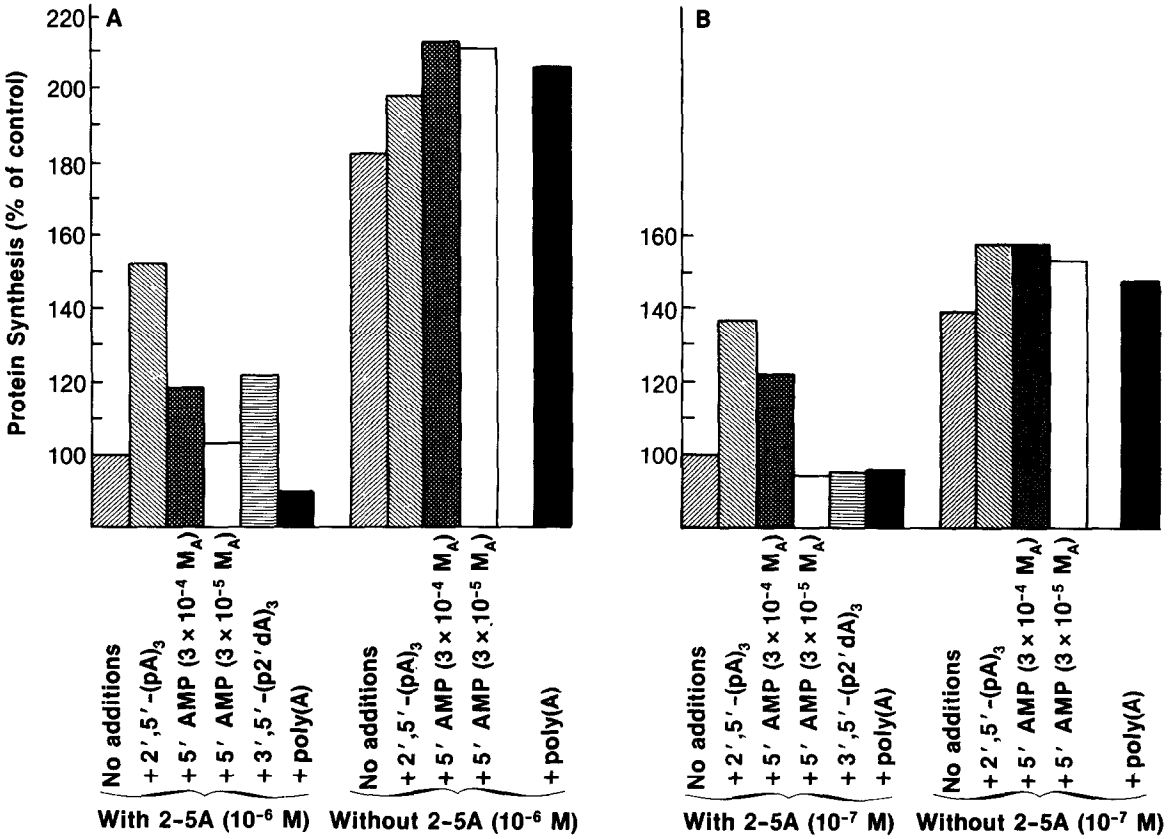

Fig.2. Effect of adenosine-containing nucleotides on inhibition of protein synthesis by 2–5 A. L cells were treated with calcium phosphate precipitates containing 2–5 A alone, or an adenosine nucleotide alone, or with 2–5 A and an adenosine nucleotide, as indicated, in 3 separate experiments. The 2–5 A was applied at a final concentration, based on adenosine residues (M_A), of 10^{-6} M_A (A) or 10^{-7} M_A (B) and the adenosine nucleotide was applied at a final M_A of 3×10^{-4} M_A (and 3×10^{-5} M_A for 5′-AMP). 100% represents the level of protein synthesis inhibition in the presence of 2–5 A alone. The results with the other nucleotides are expressed as a percentage relative to that value. In each experiment, all compounds were evaluated in quadruplicate. The values given for 2′,5′-$(pA)_3$ and 5′-AMP (3×10^{-4} M_A) also represent the average from all 3 separate experiments.

of inhibited protein synthesis. With addition of 3′,5′-(p2′dA)$_3$ and 10^{-6} M 2-5 A, the level of protein synthesis increased slightly to 122% compared to 152% with addition of 2′,5′- (pA)$_3$ and 2-5 A (10^{-6} M) (fig.2A). Furthermore, AMP, at 3×10^{-4} or 3×10^{-5} M_A, or poly(A)$_n$ at a concentration equimolar to 2′,5′-(pA)$_3$ was introduced with 2-5 A into L cells. The levels of protein synthesis were not significantly affected by cotransfection of either of the adenosine-containing compounds with 2-5 A. Multiple transfections indicated that introduction of 2′,5′-(pA)$_3$, AMP, or poly(A)$_n$ alone into mouse cells did not result in any inhibition of protein synthesis.

To evaluate the possibility that 2′,5′-(pA)$_3$ was acting nonspecifically to inhibit 2-5 A due to more efficient precipitation of 2′,5′-(pA)$_3$ than 2-5 A by calcium phosphate, an experiment was designed in which the amount of 2-5 A precipitated in the presence or absence of 2′,5′-(pA)$_3$ was compared. Radioactively labeled 2-5 A trimer triphosphate, prepared by HPLC purification of 2-5 A obtained from a 2-5 A synthetase reaction, was precipitated with or without 2′,5′-(pA)$_3$ and the amount of unprecipitated radioactivity in the supernatant of each pelleted sample was measured (not shown). Upon addition of unlabeled 2′,5′-(pA)$_3$ to 2-5 A, the amount of unprecipitated radioactivity was slightly reduced. Therefore, the presence of the antagonist in precipitation mixtures did not affect the efficiency at which the inhibitor was precipitated with calcium phosphate. This conclusion is further supported by the observation that the dose response to 2-5 A remained linear in the presence of a uniform concentration of antagonist (see fig.1).

4. DISCUSSION

Multiple responses are elicited in cells which have been exposed to interferon. As of yet, it is not clear what mechanisms are responsible for mediating the various activities of interferon. We have now shown that simultaneous introduction of 2-5 A and the corresponding 5′-monophosphate, 2′,5′-(pA)$_3$, into living cells results in prevention of the inhibition of protein synthesis by 2-5 A. The development of this system, in which the 2-5 A enzymatic pathway induced by interferon treatment can be functionally antagonized in living cells, may provide a model with which to assess the nature of the participation of this 2-5 A pathway in interferon-induced cellular and viral responses.

An additional phenomenon associated with introduction of antagonist was observed. Upon introduction of 2′,5′-(pA)$_3$ or its analogs alone into L cells, protein synthesis was significantly stimulated in a dose-dependent manner (figs 1,2A and B). This enhancement was consistently observed with 2′,5′-(pA)$_3$, 5′-AMP, and poly(A). The ability to antagonize 2-5 A-mediated inhibition of protein synthesis did not correlate with the level of enhancement of protein synthesis. This effect was not observed with the same oligonucleotides in the in vitro cell-free translation system (not shown). Evidence obtained from [^{3}H]leucine labeling of the cells to measure the extent of cell loss during the harsh transfection procedure indicated that treatment of the cells with precipitates containing the oligonucleotides may somehow stabilize the cells since the number of cells remaining after manipulations increased slightly in the presence of precipitates containing the antagonist or its analogs. Alternatively, these adenosine nucleotides, which do not inhibit protein synthesis, may participate more directly at the level of protein synthesis to mediate this enhancement phenomenon. Further investigation of the involvement of 2-5 A related oligonucleotides in stimulating protein synthesis may provide information regarding the regulation of cellular biosynthesis.

The evidence presented in this report that 2′,5′-(pA)$_3$ acts as an antagonist of 2-5 A inhibitory activity contrasts sharply with results obtained from similar experiments using intact mouse L cells by Haugh et al. [19]. Using the calcium phosphate precipitation technique to introduce oligonucleotides into L cells, these workers observed inhibition of protein synthesis by 50 μM 2′,5′-(pA)$_3$ 2 h after removal of the precipitate from the cells. Furthermore, simultaneous introduction of 50 μM 2′,5′-(pA)$_3$ with 50 nM 2-5 A (a 1000-fold difference in concentration) did not affect the level of inhibition of protein synthesis by 2-5 A. In our system, the addition of 100 μM 2′,5′-(pA)$_3$ reproducibly stimulates L cell protein synthesis by 2.25 h after removal of the precipitate. Moreover, cointroduction of 100 μM 2′,5′-(pA)$_3$ and 1 μM 2-5 A (a 100-fold difference in concentration) results in a dramatic antagonism of 2-5 A inhibitory activity. These contrasting results may reflect differences in

the length of time the cells are exposed to the potentially toxic calcium phosphate precipitate. The cells of Haugh et al. [19] were treated with the precipitate for 2 h; whereas, during this investigation, cells were incubated with precipitate for 45 min and the level of protein synthesis was determined with respect to the total number of cells present after manipulation. However, these conflicting results cannot be totally reconciled by technical differences since in vitro experiments using L cell-free protein synthesis systems have also provided results that are at odds [10,11,19, 20]. Therefore, it would be valuable to examine the effectiveness of 2′,5′-$(pA)_3$ antagonism in other systems. Miyamoto et al. [11], for instance, found that 2′,5′-$(pA)_3$ could completely block the 2-5 A-dependent degradation of reovirus RNA in mouse fibroblasts, in agreement with our earlier results [10].

In summary, 2′,5′-$(pA)_3$ was found to stimulate protein synthesis in intact cells and to prevent the translational inhibitory effects of 2-5 A. Whether there is a causal relationship between these phenomena and the 2-5 A synthetase/2-5 A-dependent endonuclease system, as it has been described thus far, remains to be determined.

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