Inhibition of protein synthesis by the cordycepin analog of (2′–5′)ppp(Ap)$_n$A, (2′–5′)ppp(3′dAp)$_n$3′dA, in intact mammalian cells

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The introduction of the cordycepin analog of (2′–5′)A$_n$, (2′–5′)ppp(3′dAp)$_n$3′dA [referred to as (2′–5′)p$_3$3′dA$_n$], into mouse L929 cells and cultured human fibroblasts resulted in a dose-dependent inhibition of protein synthesis which was comparable to the inhibition observed by (2′–5′)ppp(Ap)$_n$A [referred to as (2′–5′)p$_3$A$_n$]. The inhibition of protein synthesis by (2′–5′)p$_3$3′dA$_n$ was much more persistent than that of the naturally occurring (2′–5′)p$_3$A$_n$ following prolonged incubation of cells. Furthermore, the (2′–5′)p$_3$A$_n$ was cytotoxic to mammalian cells in culture, whereas the (2′–5′)p$_3$3′dA$_n$ was not.

Cordycepin *(2′–5′)Oligoadenylate* *Endonuclease activation*
Protein synthesis inhibition *Calcium phosphate co-precipitation*

1. INTRODUCTION

A series of oligonucleotides with the unique (2′–5′)phosphodiester linkage is synthesized by an interferon-induced enzyme, (2′–5′)oligoadenylate synthetase [1]. These (2′–5′)p$_3$A$_n$ oligomers are potent inhibitors of protein synthesis in cell-free systems and in intact cells, probably via activation of a latent endonuclease (referred to as RNase L or RNase F), which degrades mRNA as well as rRNA [2]. However, (2′–5′)p$_3$A$_n$ is metabolically unstable, being rapidly hydrolyzed by (2′–5′)phosphodiesterase [3], which limits its use as a biologically active nucleotide in intact cells. Thus, structural modifications of (2′–5′)p$_3$A$_n$ which would provide increased stability and enhanced biological activity have been actively investigated.

A number of (2′–5′)p$_3$A$_n$ analogs can be synthesized from various nucleotide triphosphates by (2′–5′)oligoadenylate synthetase which appears to possess rather a broad spectrum substrate specificity [4]. We have reported:

(i) The enzymatic synthesis of the cordycepin analog of (2′–5′)p$_3$A$_n$, (2′–5′)p$_3$3′dA$_n$, from 3′dATP, using the (2′–5′)oligoadenylate synthetase from rabbit reticulocyte lysates;
(ii) The effectiveness of the analog in the inhibition of in vitro translation and in the cleavage of VSV mRNA by the endonuclease activated in the lysates;
(iii) Its increased resistance to degradation in HeLa cell, L cell, and lymphoblast extracts [5–7].

Here, we have extended the studies with (2′–5′)p$_3$3′dA$_n$ to compare its inhibitory effect with that of (2′–5′)p$_3$A$_4$ on protein synthesis in intact mammalian cells employing the calcium phosphate co-precipitation technique.

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Abbreviations: (2′–5′)p$_3$A$_n$, (2′–5′)ppp(Ap)$_n$A; (2′–5′)p$_3$3′dA$_n$, (2′–5′)ppp(3′dAp)$_n$3′dA; endonuclease, (2′–5′)A$_n$-dependent endoribonuclease

2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]Methionine (1057 Ci/mmol) was purchased

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from New England Nuclear; minimum essential medium and calf serum were from GIBCO; $(2'-5')p_3A_3$ and $(2'-5')p_3A_4$ were from P.L. Biochemicals. $(2'-5')p_33'dA_3$ and $(2'-5')p_33'$-dA_4 were prepared by enzymatic synthesis from 3'-dATP in rabbit reticulocyte lysates as in [5–7]. The chemically synthesized $(2'-5')p_33'dA_3$ and $(2'-5')p_33'dA_4$ were generously supplied by Dr P. Torrence (NIH). The structures of enzymatically and chemically synthesized $(2'-5')p_33'dA_n$ have been reported as identical [7].

2.2. *Cell culture*

Mouse L929 cells and normal human fibroblasts (GM 731) were grown in monolayer culture in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum. Cells were seeded in 24-well plates at 1.5×10^5 cells/well one day prior to use.

2.3. *Cell treatment with $(2'-5')p_3A_n$ or $(2'-5')p_33'dA_n$ and measurement of cellular protein synthesis*

The calcium phosphate co-precipitation technique was used to introduce $(2'-5')p_3A_n$ and $(2'-5')p_33'dA_n$ into cells as in [8]. $(2'-5')p_3A_n$ or $(2'-5')p_33'dA_n$ were first mixed with $CaCl_2$, then Hepes-buffered saline (prepared from recrystallized Hepes) was added. The resulting solutions were allowed to form fine co-precipitates before being applied to the cell monolayers. The concentration of $(2'-5')p_3A_n$ or $(2'-5')p_33'dA_n$ (in 200 μl) was 10^{-7}–10^{-10} M for the first 45 min incubation at room temperature. An equal volume of serum-free medium was added to each well and incubated for 90 min at 37°C. The liquid in each well was then aspirated, [^{35}S]methionine (1057 Ci/mmol, 2 μCi/well) was added and incubated for 60 min. After 60 min, the [^{32}S]methionine incorporated into the trichloroacetic acid-precipitable material was determined [8]. All assays were done in triplicate and data are expressed as a mean obtained from 4 separate experiments.

3. RESULTS AND DISCUSSION

3.1. *Inhibitory effect of $(2'-5')p_33'dA_3$ and $(2'-5')p_33'dA_4$ on cellular protein synthesis*

Treatment of mouse L929 cells with either enzymatically or chemically synthesized cordycepin analogs of $(2'-5')p_3A_n$ (i.e., $(2'-5')p_33'dA_3$ and $(2'-5')p_33'dA_4$) resulted in a significant inhibition of protein synthesis as measured by the incorporation of [^{35}S]methionine into the trichloroacetic acid-precipitable material (about 70% inhibition at 10^{-7} M $(2'-5')p_33'dA_3$ or $(2'-5')p_33'$-dA_4) (fig.1). The inhibition by the cordycepin analogs was approximately the same as that obtained with the naturally occurring $(2'-5')p_3A_n$. As in the case of $(2'-5')p_3A_n$, the inhibitory effect of $(2'-5')p_33'dA_n$ on cellular protein synthesis was dose-dependent. Similar results were obtained when these nucleotides were introduced into cultured human fibroblasts (GM 731) (fig.2). In contrast, the 5'-dephosphorylated $(2'-5')A_n$ and $(2'-5')3'dA_n$ cores did not inhibit protein synthesis in mouse L929 or human fibroblast cells (not

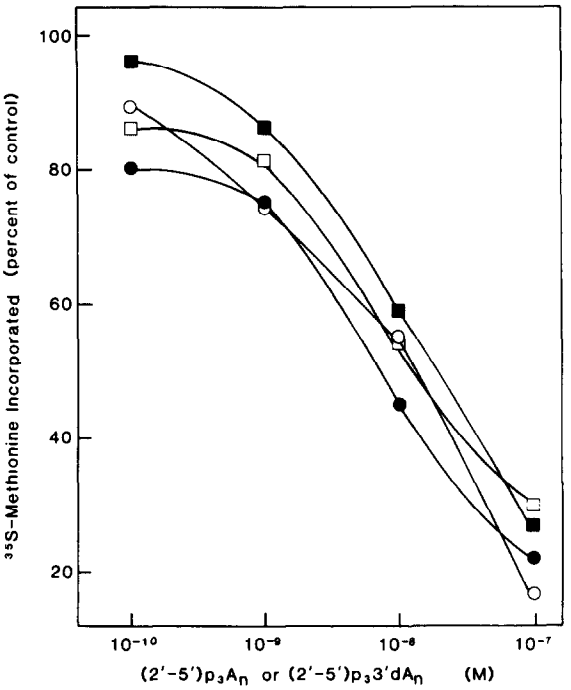

Fig.1. Inhibition of protein synthesis by $(2'-5')p_3A_n$ and $(2'-5')p_33'dA_n$ in mouse L929 cells. Inhibition of protein synthesis by $(2'-5')p_3A_3$ (●—●), $(2'-5')p_3A_4$ (○—○), $(2'-5')p_33'dA_3$ (■—■), and $(2'-5')p_33'dA_4$ (□—□) was determined in intact L929 cells using the calcium phosphate co-precipitation technique as in section 2. Control cultures were treated with calcium phosphate, but without oligonucleotides and the incorporation of [^{35}S]methionine into control cultures was taken as 100%.

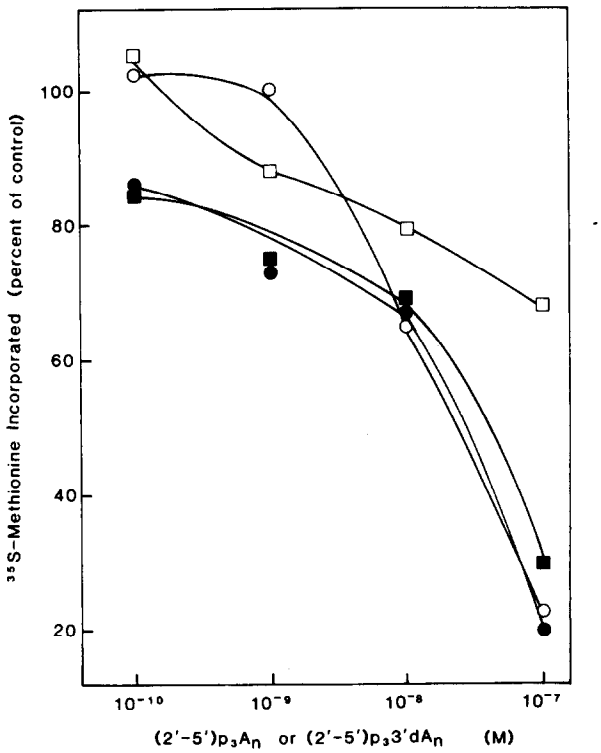

Fig.2. Inhibition of protein synthesis by (2′–5′)p$_3$A$_n$ and (2′–5′)p$_3$3′dA$_n$ in cultured human fibroblasts. Conditions for cell treatment and protein synthesis assay were identical to those in fig.1: (2′–5′)p$_3$A$_3$ (●—●); (2′–5′)p$_3$A$_4$ (○—○); (2′–5′)p$_3$3′dA$_3$ (■—■); (2′–5′)p$_3$3′dA$_4$ (□—□).

shown), in agreement with [9] on HF926 cells. (2′–5′)p$_3$3′dA$_4$ either enzymatically or chemically synthesized is as good as or a better inhibitor of protein synthesis than is (2′–5′)p$_3$A$_4$ in lysates from rabbit reticulocytes [7]. However, in intact mouse L cells (fig.1), the trimers and tetramers were equally potent in the inhibition of cellular protein synthesis, whereas the trimers appeared to be slightly more potent in human fibroblasts (fig.2).

Using mouse L cells, we have obtained evidence that (2′–5′)p$_3$A$_n$ and (2′–5′)p$_3$3′dA$_n$ are actually taken up by the cells by calcium phosphate coprecipitation (in preparation). Furthermore, the inhibition of protein synthesis observed by (2′–5′)p$_3$A$_n$ and (2′–5′)p$_3$3′dA$_n$ was probably caused by endonuclease activation; (2′–5′)p$_3$3′dA$_n$ activates the endonuclease in lysates from rabbit reticulocytes [7] and in cell-free extracts of mouse L cells (in preparation). These results show that the enzymatically and chemically synthesized cordycepin analogs of (2′–5′)p$_3$A$_n$ were as effective as (2′–5′)p$_3$A$_n$ in the inhibition of cellular protein synthesis, indicating that the replacement of the 3′-hydroxyl groups on the adenylate residue of (2′–5′)p$_3$A$_n$ with hydrogen atoms did not adversely affect its ability to inhibit protein synthesis, presumably through the activation of the (2′–5′)A-dependent endonuclease.

3.2. *The prolonged inhibition of protein synthesis by (2′–5′)p$_3$3′dA$_3$ and (2′–5′)p$_3$3′dA$_4$ upon prolonged cell incubation*

The inhibitory effect of (2′–5′)p$_3$A$_n$ on the in vitro or in vivo protein synthesis via endonuclease activation is transient, probably due to the short half-life of (2′–5′)p$_3$A$_n$ [3,10]. The continued action of the endonuclease and the prolonged maintenance of protein synthesis inhibition may require a constant presence of (2′–5′)p$_3$A$_n$ [9]. Thus, an analog of (2′–5′)p$_3$A$_n$ with increased metabolic stability, such as (2′–5′)p$_3$3′A$_n$ [5,11], may maintain its ability to inhibit protein synthesis for a prolonged period. We compared the duration of the inhibition of protein synthesis and the rate of recovery of protein synthesis of cells in cultures treated with (2′–5′)p$_3$A$_n$ and (2′–5′)p$_3$3′dA$_n$ (fig.3). When cultures were treated with either (2′–5′)p$_3$A$_n$ or (2′–5′)p$_3$3′dA$_n$ for 2.5 h, extensively washed and assayed immediately, a significant inhibition of protein synthesis (40–65%) was noted. Following the removal of the (2′–5′)nucleotides by washing and continued incubation of cells, there were marked differences in the recovery of protein synthesis in all cultures. It should be noted that shortly after the removal of the remaining (2′–5′)p$_3$A$_n$ or (2′–5′)p$_3$3′dA$_n$ in the media, the cells began to recover from the inhibition caused by (2′–5′)p$_3$A$_n$. On the other hand, the inhibition of protein synthesis by (2′–5′)p$_3$3′dA$_n$ became more pronounced after washing the cells; a maximum inhibition of protein synthesis was observed at 6 h post-treatment. Furthermore, the rate of protein synthesis recovery was much slower in cultures treated with (2′–5′)p$_3$3′dA$_n$ than in cultures treated with (2′–5′)p$_3$A$_n$. By 24 h, the cells treated with either (2′–5′)p$_3$A$_3$ (●) or (2′–5′)p$_3$A$_4$ (○) had almost completely recovered from the inhibition, whereas

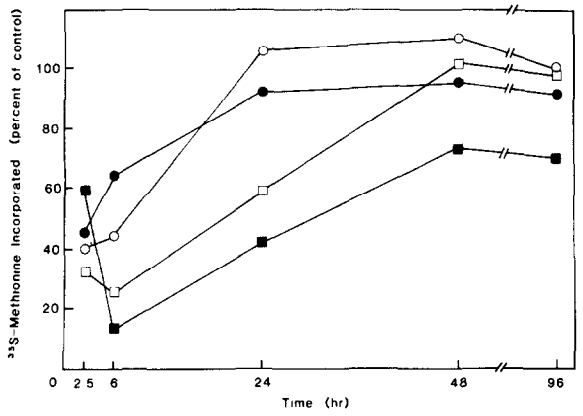

Fig.3. Kinetics of protein synthesis recovery after $(2'-5')p_3A_n$ and $(2'-5')p_3 3'dA_n$ treatment. Subconfluent mouse L929 cell cultures in 24-well plates (1.5×10^5 cells/well) were treated with $(2'-5')p_3A_n$ or $(2'-5')p_3 3'dA_n$ as in fig.1. Concentrations for all oligonucleotides used were 10^{-8} M. After incubation for 2.5 h with calcium phosphate, all cultures were extensively washed, MEM containing 10% calf serum was added, and incubation continued. At various times after the addition of calcium phosphate precipitates, protein synthesis was measured by the labeling of cells with [^{35}S]methionine for 1 h, after which the incorporation of radioactivity into trichloroacetic acid-insoluble material was determined. Level of cellular protein synthesis was calculated considering the total radioactivity incorporated per viable cell number in the culture. Cell viability was determined by trypan blue exclusion. Control cultures treated with calcium phosphate alone were assayed in parallel at each time point shown: $(2'-5')p_3A_3$ (●—●); $(2'-5')p_3A_4$ (○—○); $(2'-5')p_3 3'dA_3$ (■—■); $(2'-5')p_3 3'dA_4$ (□—□).

about 60% and 40% inhibition of protein synthesis was noted in cells treated with $(2'-5')p_3 3'dA_3$ (■) and $(2'-5')p_3 3'dA_4$ (□), respectively. Protein synthesis in cultures treated with $(2'-5')p_3A_3$, $(2'-5')p_3A_4$, and $(2'-5')p_3 3'dA_4$ returned to normal by 48 h, but a noticeable inhibition (30%) was still observed in cultures treated with the cordycepin trimer analog, $(2'-5')p_3 3'dA_3$. The inhibitory effect of $(2'-5')p_3 3'dA_3$ continued for 4 days after the initial treatment. The observed prolonged inhibitory effect of $(2'-5')p_3 3'dA_3$ is likely due to the increased stability of the cordycepin analog which can act on the $(2'-5')$A-dependent endonuclease for a longer period of time and continue to cause an extensive degradation of mRNA and/or rRNA.

During the course of these experiments, a considerable cytotoxicity of $(2'-5')p_3A_n$ was noted upon the prolonged incubation of mouse L cells. $(2'-5')p_3A_n$ resulted in about 50% cell death by 48–96 h, while there was no apparent cytotoxic effect of $(2'-5')p_3 3'dA_n$ (not shown). This observation correlates well with our finding that the naturally occurring $(2'-5')A_n$ core was cytotoxic to human lymphocytes whereas the cordycepin core analog was cytostatic [12].

We have shown that the replacement of the 3'-hydroxyl groups of the $(2'-5')p_3A_n$ molecule with hydrogen atoms does not affect the activation of the partially purified $(2'-5')$A-dependent endonuclease, hydrolysis of VSV mRNA and inhibition of protein synthesis in lysates from rabbit reticulocytes [7]. The cordycepin analog also inhibits protein synthesis in L929 cells and normal human fibroblasts. These in vivo findings verify our earlier in vitro studies that the cordycepin analogs of $(2'-5')$A inhibit protein synthesis [5–7]. It is of interest that neither the cordycepin trimer triphosphate nor the tetramer triphosphate analogs were inhibitors of translation in mouse L-cell free extracts programmed with encephalomyocarditis virus [13].

Since our original report on the enzymatic synthesis, increased metabolic stability and inhibition of protein synthesis by the cordycepin analog of $(2'-5')p_3A_n$ [14], studies have expanded in which modification of the 2'-terminus of the $(2'-5')p_3A_n$ molecule results in $(2'-5')p_3A_n$ analogs which also have increased metabolic stabilities, inhibit protein synthesis and inhibit methylation of the 5'-cap of mRNA [9,11,13,15–17]. The eventual goal of these studies would be the design of an analog of the $(2'-5')p_3A_n$ molecule that could replace the metabolically unstable $(2'-5')p_3A_n$ and be suitable for use in the treatment of virus diseases or cancer.

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REFERENCES

[1] Hovanessian, A.G., Brown, R.E. and Kerr, I.M. (1977) Nature 268, 537–540.

[2] Sen, G.C. (1982) Progr. Nucleic Acids Res. Mol. Biol. 27, 105–156.

[3] Minks, M.A., Benvin, S., Maroney, P.A. and Baglioni, C. (1979) Nucleic Acids Res. 6, 767–780.

[4] Hughes, B., Srivastava, P.C., Muse, D.D. and Robins, R.K. (1983) Biochemistry, in press.

[5] Doetsch, P.W., Wu, J.M., Sawada, Y. and Suhadolnik, R.J. (1981) Nature 291, 355–358.

[6] Suhadolnik, R.J., Doetsch, P., Wu, J.M., Sawada, Y., Mosca, J.D. and Reichenbach, N.L. (1981) Methods Enzymol. 79, 257–265.

[7] Suhadolnik, R.J., Devash, Y., Reichenbach, N.L., Flick, M.B. and Wu, J.M. (1983) Biochem. Biophys. Res. Commun. 111, 205–211.

[8] Hovanessian, A.G. and Wood, J.N. (1980) Virology 101, 81–90.

[9] Drocourt, J.L., Dieffenbach, C.W. and Ts'o, P.O.P. (1982) Nucleic Acids Res. 101, 2163–2174.

[10] Williams, K.G., Kerr, I.M., Gilbert, C.S., White, C.N. and Ball, L.A. (1978) Eur. J. Biochem. 92, 455–462.

[11] Eppstein, D.A., Marsh, Y.V., Schryver, B.B., Larsen, M.A., Barnett, J.W., Verheyden, J.P.H. and Prisbe, E.J. (1982) J. Biol. Chem. 257, 13390–13397.

[12] Doetsch, P.W., Suhadolnik, R.J., Sawada, Y., Mosca, J.D., Flick, M.B., Reichenbach, N.L., Dang, A.Q., Wu, J.M., Charubala, R., Pfleiderer, W. and Henderson, E.E. (1981) Proc. Natl. Acad. Sci. USA 78, 6699–6703.

[13] Sawai, H., Imai, J., Lesiak, K., Johnston, M.I. and Torrence, P.F. (1983) J. Biol. Chem. 258, 1671–1677.

[14] Doetsch, P., Wu, J., Shockman, G.D. and Suhadolnik, R.J. (1980) Fed. Proc. FASEB 39, 1778.

[15] Baglioni, C., D'Alessandro, S.B., Nilsen, T.W., Den Hartog, J.A.J., Crea, R. and Van Boom, H.J. (1981) J. Biol. Chem. 256, 3253–3257.

[16] Goswami, B.B., Crea, R., Van Boom, J.H. and Sharma, O.K. (1982) J. Biol. Chem. 257, 6867–6870.

[17] Imai, J., Johnston, M.I. and Torrence, P.F. (1982) J. Biol. Chem. 257, 12739–12745.