Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 291-297

INHIBITION OF VIRAL REVERSE TRANSCRIPTASE BY 2',5'-OLIGOADENYLATES

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Received April 10, 1987

Viral reverse transcriptase activity was inhibited in a concentation dependent manner by 2',5'-oligoadenylate. Kinetically this inhibition was of a mixed type where 2',5'-oligoadenylate was not strictly competitive with dTTP. The potency of inhibition was more marked in the absence than in the presence of sulfhydryl agents. 2',5'-oligoadenylate had no effect on DNA-dependent E. coli DNA polymerase and was much less active against mammalian DNA polymerases. This is the first report of reverse transcriptase inhibition by an inducible constitutive natural ligand.

A well documented mechanism for the antiviral action of interferon (IFN) involves the 2,5A synthetase-RNase L pathway. IFN induces 2,5A synthetase and synthesized 2,5A activates the latent RNase L, thereby inhibiting viral protein synthesis and growth (1-4). This mechanism is supported by accumulation of 2,5A in IFN treated cell (5, 6), by 2,5A inhibition of viral RNA synthesis and viral growth (2), by a correlation between induction of 2,5A synthetase and viral yield (7), and by preferential degradation of viral RNA over cellular RNA by 2,5A mediated RNase L in viral infected IFN treated cells (8, 9). Moreover, 2,5A inhibits cap methylation of viral mRNA which may render the message less active in viral protein synthesis (10, 11). We now report a different mechanism for the antiviral activity of 2,5A: the direct inhibition by 2,5A of the activity catalyzed by RVT, an RNA-dependent DNA polymerase of retroviruses which transcribes viral RNA into proviral DNA (12, 13).

MATERIALS AND METHODS

Materials- 2,5A, core 2,5A, poly(rA) $(dT)_{12-18}$, nonradiolabeled deoxyribonucleotide triphosphates, and purified calf thymus DNA polymerase

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The abbreviations used are: IFN, interferon; 2,5A, 2'5'-oligoadenylate; core 2,5A, nonphosphorylated trimer of 2,5A; RVT, reverse transcriptase; AMV, avian myeloblastosis virus; RAV-2, Rous associated virus 2; M-MLV, Moloney murine leukemia virus; DTT, dithiothreitol.

0006-291X/87 $1.50

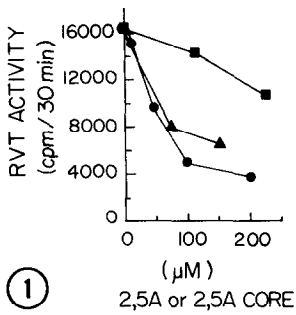
α were purchased from Pharmacia (Piscataway, NJ). [Methyl-1-^{3}H]thymidine-5'-triphosphate (40-70 Ci/mmol) was a product of ICN (Irvine, CA). Nuclease free bovine serum albumin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Purified RVT was purchased from the following sources: AMV RVT, Pharmacia (Piscataway, NJ) and Molecular Genetic Resources (Tampa, FL); RAV-2 RVT, Amersham (Arlington Heights, IL); and cloned M-MLV RVT, Bethesda Research Laboratories (Gaithersburg, MD). Calf thymus DNA and bovine pancreatic DNase I were purchased from Sigma Chemical Co. (St. Louis, MO). Purified E. coli DNA polymerase was a product of Worthington (Freehold, NJ). A mixture of various sized 2,5A (di- to hexamers, > 73% trimers or higher) was synthesized by incubating nuclei isolated from R3230AC rat mammary tumor (14) in a solution assay (15, 16). The 2,5A mixture was freed of salt by passing it through a Sephadex G25 column (1 x 90 cm, equilibrated with water) and lyophilizing. The concentration was estimated equivalent to 2,5A trimer on the basis of A_{260nm} (1 unit=28 nmoles). This preparation was resistant to digestion by RNase T_2 (17) and yielded a 60% inhibition of protein synthesis at 200 nM in a globin mRNA primed reticulocyte system (18). The DNA polymerase α was purified from R3230AC rat mammary tumor (19). The DNA polymerase β was partially purified from the same tumor using a method described by Mechali et al. (20).

RVT assay- The RVT assay mixture was according to Houts et al. (21) in which a total volume of 50 µl contained 50 mM Tris-HCl, pH 8.3; 6 mM $MgCl_2$; 40 mM KCl; 100 µg bovine serum albumin/ml; 0.5 mM [^{3}H]dTTP containing 1 µCi; 5 µg poly(rA) $(dT)_{12-18}$; appropriate amounts of RVT and various amounts of 2,5A (0-200 µM), analogs, or same volumes of water in which 2,5A was dissolved. The reaction was initiated by adding [^{3}H]dTTP after preincubating all other mixtures for 10 min at 37°C and continued incubating for 30 min at same temperature. The reactions were stopped by adding 10 µl 10 mM dTTP and put on ice-water bath. Forty-five µl of the reaction mixture were spotted onto a DE81 disc, washed four times with 5% Na_2HPO_4, twice with water, once with 95% ethanol, and dried (19). The radioactivity was counted using 5 ml of Econofluor (Dupont).

DNA polymerase assay- A previously described method (19, 22) was used. Briefly, in a final volume of 100 µl reaction mixture contained 20 mM potassium phosphate, pH 7.2; 0.1 mM EDTA; 1 mM DTT or an equivalent volume of H_2O; 250 µg/ml bovine serum albumin; 10 mM $MgCl_2$; 200 µg/ml activated (by DNase I) calf thymus DNA; 20 µM each of dATP, dCTP, dGTP; 20 µM [^{3}H]dTTP containing 0.9 µCi; appropriate amounts of enzymic solution and 2,5A or water. The reaction was initiated by adding the deoxynucleotide mixture following a 10 min preincubation at 37°C of all other ingredients and the incubation continued for 30 min. The reaction was stopped by adding 10 µl of 10 mM dTTP and put in an ice-water bath. Eighty µl of each mixture were spotted on a DE81 disc, washed, and counted as described in the RVT assay above.

RESULTS

When various amounts of 2,5A, either the trimer obtained from a commercial source or the synthesized mixture of different lengths, were included in assays of avian myeloblastosis virus (AMV) RVT, there was a concentration dependent decrease in [^{3}H]dTMP incorporation. The inhibition was noted at 10 µM 2,5A and was pronounced at 50 µM or more (Fig. 1). Inhibition of RVT by 2,5A was maximal within 15 minutes (not shown). When the kinetics of inhibition were examined by two different plots of RVT activity and dTTP concentrations (23), the inhibition was



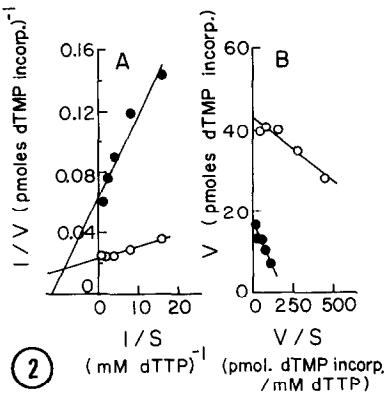
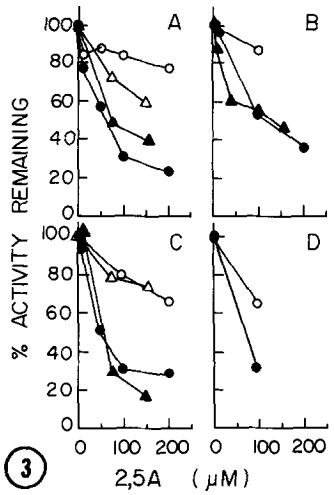


Figure 1. The concentration dependent inhibition of AMV RVT by commercial 2,5A trimers (▲–▲), 2,5A synthesized mixture by R3230AC rat mammary tumor nuclei (●–●), and the 2,5A core (■–■).

Figure 2. Kinetics of inhibition of AMV RVT by 2,5A. Varying concentrations of dTTP were incubated with a 50 μM 2,5A (synthesized mixture) for 30 min at 37°C and amounts of incorporated [^{3}H]dTMP were determined as in the RVT assays. Panel A: The Lineweaver-Burk plot, Panel B: an alternative single reciprocal plot showing a mixed-type inhibition (22). The symbols are ○, no addition of 2,5A and ●, with 2,5A.

shown to be of mixed type (Fig. 2A and B). Core 2,5A also inhibited RVT activity, but at a much reduced rate, i.e. 65% of the enzyme activity remained at 200 μM 2,5A core concentration (Fig. 1). An essentially similar inhibition was observed in each of 4 RVT enzymes from different viral sources: two purified from AMV, one from Rous associated virus (RAV-2), and one from cloned Moloney murine leukemia virus (M-MLV) (Fig. 3A-D). The average inhibition of AMV RVT with 100 μM 2,5A from different experiments was 80 ± 5 % (n=3) and 84 ± 5 % (n=7) respectively by commercial and synthesized 2,5A. Using synthesized 2,5A at 100 μM the average inhibition of RAV-2 and M-MLV was respectively 69 ± 3 % (n=4) and 45 ± 9 % (n=4). Inhibition was most dramatic in the absence of sulfhydryl reagents and was reduced when either DTT or an equivalent amount of β-mercaptoethanol was included in the reaction (Fig. 3A-D). Partial protection of 2,5A inhibition of RVT was detected as low as 0.1 mM DTT but significant protection occurred at 1 mM or above, as shown in Fig. 3A-D.

2,5A concentrations of 50 μM or even higher concentrations were relatively inactive against bacterial and mammalian DNA polymerases either in the presence or absence of sulfhydryl reagents. E. coli DNA polymerase activity was unaffected by 2,5A concentrations up to 200 μM (Fig. 4A). Calf thymus DNA polymerase α was also uninhibited by 2,5A (Fig. 4B). Limited inhibition occurred with DNA polymerase α from the R3230AC mammary tumor when enzyme activity was low (Fig. 4C). With DTT present and at a very low polymerase activity level, DNA polymerase β from R3230AC mammary tumor was unaffected by 2,5A (Fig. 4D).



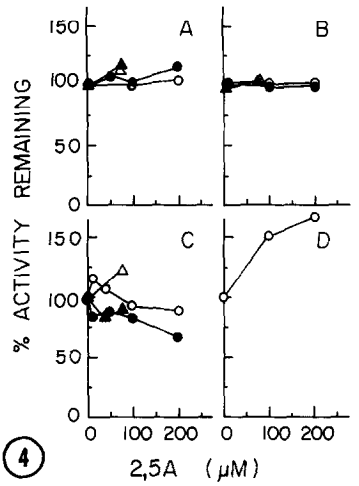


Figure 3. Effects of 2,5A on RVT from various viral sources. Varying concentrations (0-200 μM) of 2,5A, either commercial 2,5A trimer (▲,△) or 2,5A synthesized mixture (●,○), were added to RVT assays with 1 mM DTT (△–△); (○–○) and without 1 mM DTT (▲–▲ , ●–●) present in the incubation. The RVT used and the non-inhibited enzymic activity representing 100% activity were as follows: Panel A, 16,742 and 16,333 cpm with and without 1 mM DTT respectively by 0.25 units of purified AMV RVT; Panel B, 7,433 and 9,117 cpm with and without 1 mM DTT respectively by 0.5 units of AMV of different batch; Panel C, 13,600 and 15,790 cpm with and without 1 mM DTT respectively by 0.25 units of purified RAV-2 RVT; and Panel D, 14,850 and 16,487 cpm with and without 1 mM DTT respectively by 0.25 units of cloned M-MLV.

Figure 4. Effects of 2,5A on bacterial and mammalian DNA polymerases. Varying concentrations (0-200 μM) of either commercial 2,5A (▲,△) or 2,5A synthesized mixture (●,○) were included in DNA polymerase assays with (△,○) and without 1 mM DTT (▲,●) present. The sources of DNA polymerases and the non-inhibited enzymic activity respresenting 100% activity are as follows: Panel A, 2,040 and 2,333 cpm respectively with and without 1 mM DTT by purified E. coli DNA polymerase; Panel B, 18,600 and 19,600 cpm respectively with and without 1 mM DTT by purified calf thymus DNA polymerase α; Panel C, 4,618 and 2,000 cpm respectively with and without 1 mM DTT by the purified R3230AC mammary tumor DNA polymerase α; and Panel D, 616 cpm with 0.5 mM DTT by R3230AC mammary tumor DNA polymerase β.

DISCUSSION

Historically, the role of cellular 2,5A in antiviral mechanisms has been limited to systems affecting viral protein synthesis through inhibition of cellular enzymes as exemplified by the 2,5A-mediated RNase L pathway (1-4) or by 2,5A inhibition of viral mRNA methylation (10, 11). A new dimension has now been revealed with this documentation that 2,5A directly affects proviral DNA synthesis. Various inhibitors of RVT previously studied inhibited non-selectively both viral and cellular DNA polymerases (12, 13, 24, 25), conferring a cytotoxic potential. By contrast, the 2,5A inhibition we observed was selective for viral RNA-dependent DNA polymerase with little effect on various cellular DNA polymerases. Although RVT has been shown to be sensitive to other natural

agents, such as pyridoxal 5'-phosphate (26), the present report offers the first instance where an inducible cellular component inhibits RVT.

The mixed type inhibition of RVT activity by 2,5A suggests that cellular dTTP levels may be less important than the cellular concentration of 2,5A. For significant inhibition of RVT 10 μM or more of 2,5A was required. This potency is comparable to the 2,5A concentration required for inhibition of vaccinia virus mRNA (guanine-7) methyltransferase (10, 11). It was higher than that required for inhibition of protein synthesis in vitro mediated by RNase L degration of RNA (1-4), but was similar to the concentration of 2,5A required for inhibition of protein synthesis in intact cells (2, 27). Thus far constitutive 2,5A has been found only in submicromolar concentrations in the cells or tissues examined (6, 28-32). In interferon treated cells, 2,5A concentrations reached 3-5 μM (30, 33). However, in light of evidence for preferential degradation of viral RNA over cellular RNA by 2,5A-mediated activation of RNase L (8, 9), it may be possible for a cell-specific and intracellularly localized induction of 2,5An synthesis to occur. During peak infection and during viral RNA replication at the vicinity of RVT and viral RNA, which is thought to activate 2,5A synthetase, the 2,5A concentrations might exceed those required for RVT inhibition.

Partial protection conferred by sulfhydryl reagents against 2,5A inhibition of RVT is intriguing. Since DTT is partially protected at a concentration as low as 0.1 mM, it seems most likely that 2,5A inhibition of RVT involved interaction with an SH group at the active site. This is similar to the protection of AMV RVT inhibition by a synthetic inhibitor, captan (34). Thus, the inhibitory effect of 2,5A on RVT in vivo depends not only on 2,5A concentrations in the natural environment, but also on the availability of endogenous SH groups, most notably those on glutathione. Since data suggest that both the promotion of 2,5A synthesis by the replicative intermediates and the degradation of nascent viral transcripts by 2,5A activated RNase L occur in a localized manner (8,9), we suspected 2,5A inhibition of RVT may occur in a localized way. Whether this localized action can escape the interference by the high concentration of gluthathion in the cells remains to be investigated.

In summary we present evidence that 2,5A is a specific anti-RVT agent, thus providing a hitherto unrecognized pathway of 2,5A action. Since RVT from various animal viruses was sensitive to 2,5A, it is likely that RVT from human retroviruses may also be sensitive to 2,5A. Inhibitory effects of 2,5A on both viral protein synthesis and viral RNA transcription may explain in part the potent antiviral action of interferon. Selective

inhibition of viral, but not cellular DNA polymerases suggests that the present mechanism may aid in the development of therapeutically useful anti-viral 2,5A analogs.

ACKNOWLEDGEMENTS

This investigation was supported by Grant CA35916 from the National Cancer Institute. We are grateful to Kathleen A. Maguire for the determination of 2,5A inhibition of cell-free protein synthesis and to Drs. Elliot S. Vesell and John D. Connor for their critical reading of the manuscript.

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