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SELECTIVE INHIBITION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE BY DIADENOSINE 5',5''' -P^1,P^4-TETRAPHOSPHATE

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SUMMARY

Diadenosine 5',5''' -P^1,P^4-tetraphosphate ($A_{p4}A$) was found to strongly and specifically inhibit the activity of terminal deoxynucleotidyl transferase (TdT) from calf thymus, whereas $A_{p4}A$ had no remarkable effect on the activities of mouse DNA polymerases α, β and γ. Kinetic studies revealed that the inhibition of TdT by $A_{p4}A$ was due to competition with substrate deoxynucleoside triphosphate(s). Thus, $A_{p4}A$ might block the substrate binding site of TdT.

INTRODUCTION

Diadenosine 5',5''' -P^1,P^4-tetraphosphate ($A_{p4}A$) has been shown to occur in both prokaryotic and eukaryotic cells as a product of the backreaction of amino acid activation step in protein synthesizing system (1,2,3,4) and this compound was suggested to act as a positive signal for mammalian cell growth (4). The intracellular concentration of $A_{p4}A$ fluctuates depending on the proliferation rate of mammalian cells (4). Furthermore, the addition of $A_{p4}A$ to permeabilized G_1-arrested baby hamster kidney cells induced to initiate DNA replication (5). Recently, Grummt et al. reported that $A_{p4}A$ specifically binds to one of the 7 polypeptide subunits (Mr 57,000) of calf thymus DNA polymerase α (6). This result suggests that DNA polymerase α is an intracellular target of $A_{p4}A$ and support the hypothesis that $A_{p4}A$ is an intracellular signal molecule for the initiation of DNA replication. However, effect of $A_{p4}A$ on the function(s) of DNA polymerase α is still unknown.

This paper describes that $A_{p4}A$ has no remarkable effect on nucleotide-polymerizing activity of DNA polymerase α as well as on the activities of DNA polymerases β and γ from mouse myeloma. On the other hand, it was found that

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the activity of terminal deoxynucleotidyl transferase (TdT) from calf thymus was specifically inhibited in the presence of $A_{p4}A$.

MATERIALS AND METHODS

Chemicals. Tritiated deoxynucleoside triphosphates (dNTP's) were purchased from Radiochemical Centre, Amersham, England. Unlabeled dNTP's and $(dT)_{12-18}$ were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany. $A_{p4}A$ and all other poly- and oligo-nucleotides were the products of P-L Biochemicals, Inc., Milwaukee, Wis. Calf thymus DNA from Sigma Chemical Co., St. Louis, Mo., was activated according to the method of Schlabach et al. (7). DEAE-cellulose paper (DE81, ϕ23mm) was from Whatman Ltd., Springfield Mill, Maidstone, Kent, England.

DNA polymerases α, β and γ and TdT. DNA polymerases α, β and γ were purified from mouse myeloma MOPC104E cells as previously described (references 8,9 and 10 for α, β and γ, respectively), and the preparations at the step of DNA-cellulose column chromatography were used throughout the present studies. TdT was purified from calf thymus to homogeneity. Purification procedures will appear elsewhere.*

Assays for DNA polymerases and TdT. The activity of DNA polymerase α was assayed with activated calf thymus DNA or $(dT)_n \cdot (rA)_{12-18}$ as the template·primer. The activity of DNA polymerase β was assayed with activated calf thymus DNA and that of DNA polymerase γ with $(rA)_n \cdot (dT)_{12-18}$ or activated calf thymus DNA. The activity of TdT was assayed with $(dA)_{12-18}$ as the primer and [^{3}H]dATP or [^{3}H]dCTP as the nucleotide substrate. All assay conditions optimized for activities are summarized in Table 1. Reactions of 50 μl were started by adding 1-5 μl enzyme and incubations were carried out at 37° for 30 min. The reaction was stopped by adding 15 μl of 0.2 M EDTA and immersing the mixture in ice. Then 50 μl aliquot was transferred to DE81 filter disc and processed for counting radioactivity as described previously (11). The concentrations of [^{3}H]dNTP and $A_{p4}A$ were varied in experiments on the Km, Ki and mode of inhibition. Km and Ki values were determined by Lineweaver-Burk and Dixon plots, respectively. In all cases of the kinetic experiments, the incorporation of [^{3}H]dNMP was proportional to the incubation time.

RESULTS

Effect of $A_{p4}A$ on the activities of DNA polymerases α, β and γ and TdT.

Effect of $A_{p4}A$ on the activities of DNA polymerases α, β and γ and TdT was examined. As shown in Fig. 1A, $A_{p4}A$ did not show any remarkable effect on the nucleotide-polymerizing activity of DNA polymerase α with any of the template·primers tested; with activated calf thymus DNA (incorporation of [^{3}H]dATP or [^{3}H]dTTP) or with $(dT)_n \cdot (rA)_{12-18}$ (incorporation of [^{3}H]dATP). No inhibition or activation by $A_{p4}A$ was also observed in the case of DNA polymerase β and γ except that $(rA)_n \cdot (dT)_{12-18}$ -directed activity of DNA

* Nakamura, H., Morita, T. and Yoshida, S.,submitted for publication.

Table 1. Assay conditions for various DNA polymerases[a]

DNA polymerase	Template·primer or primer	Reaction components and their concentrations: Template·primer or primer concentration[b] (μg/ml)	Tris buffer: pH	Tris buffer: Concentration (mM)	[^{3}H]dNTP and concentration (μM)	Unlabeled dNTP concentration (μM)	Divalent cation and concentration (mM)	Monovalent cation and concentration (mM)	DTT[c] concentration (mM)
α	Activated calf thymus DNA	80	8.5	50	dATP or dTTP 10	Other 3 dNTP's, 10 each	Mg^{2+}, 4		1
	$(dT)_n \cdot (rA)_{12-18}$	10(5:1)[d]	7.5	50	dATP, 10		Mn^{2+}, 1	K^+, 40	1
β	Activated calf thymus DNA	200	9.0	100	dTTP, 10	Other 3 dNTP's, 10 each	Mg^{2+}, 10	K^+, 30	5
γ	Activated calf thymus DNA	100	7.5	100	dTTP, 1	Other 3 dNTP's, 10 each	Mg^{2+}, 5	K^+,150	5
	$(rA)_n \cdot (dT)_{12-18}$	10(10:1)	7.5	100	dTTP, 1		Mn^{2+}, 0.1	K^+, 70	5
TdT	$(dA)_{12-18}$	6	8.0[e]	100[e]	dATP, 10		Mn^{2+}, 0.1	K^+, 10	1
					dCTP, 10		Mn^{2+}, 0.5	K^+, 30	1

[a] All reaction mixtures contained 15% (v/v) glycerol and 400 μg/ml bovine serum albumin.

[b] Concentration with respect to the template in the case of synthetic homopolymer duplex.

[c] DTT, dithiothreitol.

[d] Numbers in parentheses, base ratios of template to primer.

[e] N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was used instead of Tris buffer.

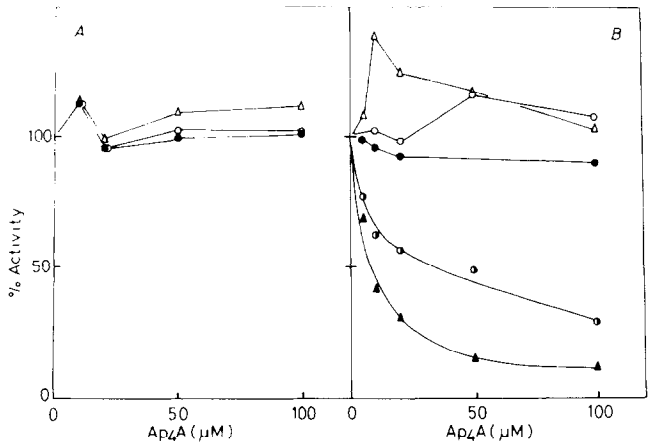

Fig. 1. Effect of $A_{p4}A$ on the activities of DNA polymerases α, β and γ and TdT. Concentrations of $A_{p4}A$ are indicated in the figures. (A) DNA polymerase α activity was measured by determining incorporation of [^{3}H]dTMP (●) or [^{3}H]dAMP (○) with activated calf thymus DNA. DNA polymerase α activity was also measured by determining incorporation of [^{3}H]dAMP with $(dT)_n \cdot (rA)_{12-18}$ (△). (B) DNA polymerase β activity was measured by determining incorporation of [^{3}H]dTMP with activated calf thymus DNA (●), and DNA polyemrase γ activity by incorporation of [^{3}H]dTMP with $(rA)_n \cdot (dT)_{12-18}$ (○) or with activated DNA (△). TdT activity was measured by determining incorporation of [^{3}H]dCMP (▲) or [^{3}H]dAMP (◑) with $(dA)_{12-18}$ as the primer. All assay conditions are described in "Materials and Methods". The specific radioactivities of [^{3}H]dNTP's were 600 cpm/pmol for DNA polymerase α and β, 6,000 cpm/pmol for DNA polymerase γ and 200 cpm/pmol for TdT. One hundred % values (pmol) were as follows: in A, 24.8 (●), 17.4 (○) and 39.0 (△); in B, 2.4 (●), 11.5 (○), 1.4 (△), 42.9 (▲), and 33.5 (◑).

polymerase γ was slightly stimulated by low concentrations of $A_{p4}A$ (40% activation by 10 μM $A_{p4}A$) (Fig. 1B). In contrast to DNA polymerases α, β and γ, the activity of TdT was strongly inhibited by $A_{p4}A$ especially when assayed by incorporation of [^{3}H]dCTP (Fig. 1B). Inhibition of TdT activity was also observed but to a lesser extent when [^{3}H]dATP was used (Fig. 1B).

Effect of $A_{p4}A$ on the nucleotide-polymerizing activities of DNA polymerases α, β and γ and TdT was also examined by preincubating these polymerases for 10 min with $A_{p4}A$ prior to assay and almost the same results as described above were obtained. Furthermore, inability of $A_{p4}A$ to serve as a primer for TdT was confirmed (data not shown). Therefore, $A_{p4}A$ was concluded to be a specific inhibitor of TdT which reversibly binds to the TdT molecule.

Mode of inhibition of TdT by $A_{p4}A$ and determination of inhibition constant(s). As seen in double reciprocal plot, the Km of TdT for dCTP was

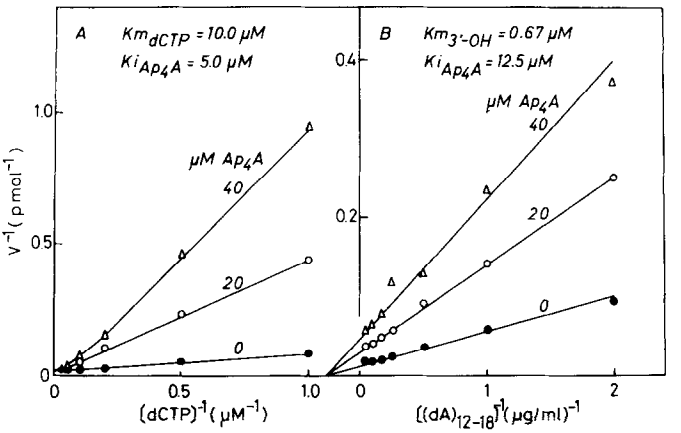

Fig. 2. Analysis of inhibition of TdT by $A_{p4}A$. Reactions were carried out with $(dA)_{12-18}$ and [^{3}H]dCTP (180 cpm/pmol in A and 400 cpm/pmol in B) under the assay conditions described in "Materials and Methods", except that various concentrations of [^{3}H]dCTP (A) and $(dA)_{12-18}$ (B) were used in the presence of 0, 20, 40 μM $A_{p4}A$. The figures represent double reciprocal plots.

determined to be 10.0 μM and the mode of inhibition of TdT by $A_{p4}A$ was competitive to dCTP (Fig. 2A). Ki value of TdT for $A_{p4}A$ was estimated to be 5.0 μM from Dixon plot. On the contrary, the mode of inhibition of TdT by $A_{p4}A$ was noncompetitive with respect to the primer, $(dA)_{12-18}$ (Fig. 2B). In this case, Ki for $A_{p4}A$ was determined to be 12.5 μM which is much higher than Km for 3'-OH of the primer, $(dA)_{12-18}$ (0.67 μM**). Similar results on the mode of inhibition by $A_{p4}A$ were obtained when [^{3}H]dATP was used as substrate. These results indicate that $A_{p4}A$ binds to the site of dNTP which is different from that of the primer.

DISCUSSION

Grummt suggested that $A_{p4}A$ stimulated the initiation of DNA synthesis in quiescent mammalian cells (5). Recently, it was demonstrated by Grummt et al. that DNA polymerase α was a target of $A_{p4}A$ and that a polypeptide subunit (Mr 57,000) was the $A_{p4}A$-binding constituent of calf thymus DNA polymerase α (6). From these results one can presume that DNA polymerase α which seems to

** The concentration of 3'-OH was calculated assuming that the nucleotide number of the primer $(dA)_{12-18}$ was 15, the average of 12 and 18.

be responsible for DNA replication is activated by binding of $A_{p4}A$ and consequently triggers initiation of DNA synthesis in the cell. However, our results showed that the nucleotide-polymerizing activity of DNA polymerase α was not stimulated by the addition of $A_{p4}A$ under any of the assay conditions employed. This strongly suggests that $A_{p4}A$-mediated induction of DNA synthesis in the cell is not due to direct effect of this compound on the polymerase activity but probably due to indirect effect on DNA synthesis through some unknown mechanism(s).

In contrast to DNA polymerases α, β and γ, the activity of TdT was strongly inhibited by $A_{p4}A$ (Fig. 1B). Kinetic experiments clearly revealed that the inhibition was due to competition between nucleotide substrate and $A_{p4}A$ on the same substrate binding site of TdT (Fig. 2A). Lower Ki value for $A_{p4}A$ than Km for dCTP is indicative of higher affinity of TdT for $A_{p4}A$. Our result that $A_{p4}A$ binds to the dNTP-binding site of TdT is quite distinct from the results that the catalytic and the $A_{p4}A$ binding sites of DNA polymerase α are not identical (6). On the other hand, the inhibition by $A_{p4}A$ was noncompetitive with respect to $(dA)_{12-18}$, indicating that the binding site for the primer is different from that for dNTP (Fig. 2B). Presence of separate binding site for nucleotide substrate and for the primer was also suggested in the study of Mn^{2+}-dATP mediated inhibition of TdT (12).

Biological function of TdT is still unknown. Baltimore proposed that TdT may be a somatic mutagen and may be responsible for the generation of immunoglobulin diversity (13). Since then no experimental evidence concerning this was presented and this interesting hypothesis remains unproven. However, the differential susceptibility of TdT versus other replicative DNA polymerases to $A_{p4}A$ as well as to ATP (14) and dATP (12) may suggest that different regulatory mechanism for TdT is functioning in the cells which contain this enzyme.

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