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AN INHIBITOR OF REVERSE TRANSCRIPTASE IN THE CYTOPLASM OF THE CULTURED CELLS AND THE SPLEEN OF MICE

Makoto Rokutanda, Yukiko Y. Maeda and Sumiyo T. Watanabe

Department of Biochemistry, Tokyo Metropolitan Institute of Medical Science, Bunkyoku, Tokyo 113, Japan

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An inhibitor which inhibits reverse transcriptase of Moloney leukemia virus was found in the cytoplasm of the cultured cells and the spleen of mice. It had a molecular weight of 85,000 based on its rate of sedimentation in glycerol gradients. It inhibits the reverse transcriptase of Moloney leukemia virus and E. coli DNA polymerase I when poly rA·oligo dT is used as template-primer, but does not inhibit when poly dA·oligo dT is used. Enzymatic activities of the inhibitor were examined. RNase, DNase and phosphatase activities are so low that the inhibitory activity does not result from them. Protease treatment of the inhibitor shows that it is protein in nature. It is shown by pretreatment of the inhibitor with the reverse transcriptase that the inhibitor does not inactivate the enzyme directly.

INTRODUCTION

Reverse transcriptase (EC. 2.7.7.7.) transcribes viral RNA into DNA at the early stage of infection of oncorna virus. The enzyme is synthesized in virus-infected cells, incorporated into the virus particles and starts to synthesize DNA after infection of permissive cells with the virus. Presence of reverse transcriptase in non virus producing infected cells and normal cells has also been reported (1). There should be some mechanism of regulation of activity of reverse transcriptase in the cells.

We reported previously (2) that reverse transcriptase found in the cytoplasm of the virus producing cells had a higher molecular weight than that of the virus particles. We consider that transition of the high molecular form to the low molecular form is one of the mechanisms regulating reverse transcriptase. Phosphorylation of the reverse transcriptase

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by protein kinase controls the activity of the enzyme (3,4). We found an inhibitor of reverse transcriptase which does not inhibit DNA dependent DNA polymerase of E. coli, in the cytoplasm of the cultured cells and spleen of the mice. We consider that this is another mechanism regulating the activity of the reverse transcriptase in the cells.

MATERIALS AND METHODS

Cells. HeLa and 78A-1 (5) cells were propagated in roller bottles, which were rotated at 0.5-1.0 rpm. Eagle's minimum essential medium enriched with two fold the normal concentration of aminoacids and vitamins was used for tissue culture with 10 % heat inactivated calf serum. The cultured cells were harvested by trypsin treatment and washed 3 times with phosphate buffered saline without Mg^{++} and Ca^{++}.

Cytoplasmic fractions. The cultured cells ($2\text{-}4 \times 10^9$ cells in 4-6 roller bottles) were harvested by trypsinization, washed with phosphate buffered saline, and suspended in 10 volumes of 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5 % NP-40 and 10 mM 2-mercaptoethanol. The cells were kept for 20 min. in an ice bath, broken with a Dounce homogenizer and centrifuged at 1,000 xg for 10 min. The supernatant was collected and stored at -80 °C until use. Mouse spleens were homogenized in Potter-Elvehjem homogenizer with Teflon pestle turning at 2,000 rpm and centrifuged at 1,000 xg for 10 min. and 10,000 xg for 20 min. as described above.

Reverse transcriptase. Purification of reverse transcriptase of the virions of Moloney leukemia virus was described previously (2).

Assay of inhibitory activity of the inhibitor. The cytoplasmic fractions and the purified reverse transcriptase were mixed, incubated for 30 min. at 37 °C and activity of the reverse transcriptase was assayed as described before (2). As control, the assay was performed without reverse transcriptase or inhibitor. Most experiments in this paper were done with the inhibitor prepared by glycerol gradient centrifugation.

Glycerol gradient centrifugation of the inhibitor. Glycerol gradient centrifugation of the inhibitor was performed as descibed below. 5-20 % (V/V) glycerol gradients were prepared in 10 mM Tris-HCl pH 7.4, 0.1 M KCl, 10 mM 2-mercaptoethanol and 0.2 % NP-40. Glycerol gradient columns were centrifuged at 40,000 rpm for 17 hours in a Beckman SW 50.1 rotor. After fractionation of the gradient columns (200 ul/fraction), 10-20 units of reverse transcriptase or E. coli DNA polymerase I were added to each fraction. After incubation at 37 °C for 30 min., the activity of the reverse transcriptase was assayed.

RESULTS AND DISCUSSION

Inhibition of reverse transcriptase activity by the inhibitor. The inhibitory activity of the cytoplasmic fractions of the cultured cells and the spleen of mice was assayed as described in Materials and Methods. Almost 98-100 % of the activity of the reverse transcriptase was inhibited by treatment with the extracts (Table 1). In our assay condition of the reverse transcriptase, the cytoplasmic fractions without the reverse

Table 1. Inhibition of reverse transcriptase by cytoplasmic fractions*

Source of inhibitor	Assay with reverse transcriptase**	Assay without reverse transcriptase
HeLa	426.3***	345.1
78A-1****	347.3	291.6
KHOS-NP*****	1,351.0	964.0
DBA/2 spleen	288.7	282.7
C_{57}BL/6 spleen	391.8	440.5
NIH swiss spleen	403.4	475.8
-	15,949.6	-

One unit of reverse transcriptase or E. coli DNA polymerase I will catalyze the incorporation of 10 picomoles of dTTP into an acid insoluble product in 30 min. at 37°C, using poly rA·oligo dT or poly dA·oligo dT as template-primer respectively.
One unit of inhibitor will inhibit one unit of reverse transcriptase.

* 67.3 ug protein/reaction was used.
** 10 units of reverse transcriptase were used.
*** cpm
**** See reference 5.
***** See reference 8.

transcriptase almost could not incorporate dTTP into an acid insoluble form when poly rA·oligo dT was used as template-primer at presence of Mn++.

Glycerol gradient centrifugation of the inhibitor. Glycerol gradient centrifugation of the inhibitor (Fig. 1) shows that molecular weight of the inhibitor is about 85,000. In order to examine the specificity of the inhibition, the inhibitory activity of the cytoplasmic fraction of HeLa cells against E. coli DNA polymerase I was examined. As shown in Fig. 1, when poly rA·oligo dT was used as template-primer, inhibition of the enzyme activity was observed but when poly dA·oligo dT was used, no inhibition was observed. E. coli DNA polymerase I is able to incorporate dTTP into an acid insoluble form when poly rA·oligo dT is used as template-primer though the incorporation with the former is lower than that with the latter. It was concluded that the inhibitor was effective when poly rA·oligo dT was used as template-primer. When poly dA·oligo dT was used as template-primer, the reverse transcriptase purified from Moloney

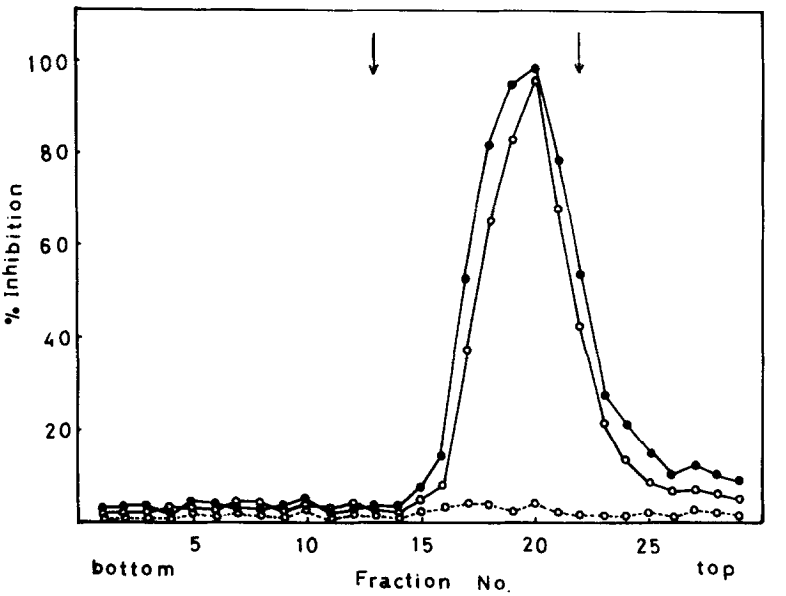

Fig. 1. Glycerol gradient centrifugation of inhibitor of reverse transcriptase.
Moloney leukemia virus reverse transcriptase was assayed with poly rA·oligo dT as template-primer (●——●).
E. coli DNA polymerase I was assayed with poly rA·oligo dT (○——○) and poly dA·oligo dT (○---○) as template-primer.
Arrows show the position of bovine serum albumin (right) and human gamma globulin (left). Left side of the figure is bottom of the gradient.

leukemia virus produced by 78A-1 cells, has 0.1-0.5 % of the activity assayed with poly rA·oligo dT, so, we could not examine the effect of the inhibitor on the reverse transcriptase when poly dA·oligo dT was used as template-primer.

Enzymatic activities of the inhibitor. RNase, DNase and phosphatase activities of the inhibitor partially purified by glycerol gradient centrifugation were examined, because these enzymatic activities affect the activity of the reverse transcriptase by destruction of template, primer, products and substrates. Table 2 shows that these activities were slight, though the inhibition was almost 100 %.

Treatment of the inhibitor with proteases. In order to examine the nature of the inhibitor, the inhibitor was treated with proteases. As shown in Table 3, the activity of the inhibitor was inactivated by proteinase K and trypsin treatment. It is suggested the inhibitor is protein in nature.

Table 2. Enzymatic activities of reverse transcriptase inhibitor*

Enzyme	Activities** (%)
RNase***	3.1
RNase H****	1.9
DNase*****	2.3
Dephosphorization of thymidine triphosphate	4.2
Destruction of Poly A	1.4
Inhibition of reverse transcriptase	100.0

* The inhibitor was centrifuged in glycerol gradients. 20 units of the inhibitor were used (8.2 ug protein/reaction).

** $\frac{\text{no inhibitor} - \text{inhibitor}}{\text{no inhibitor}} \times 100$

*** ^{14}C-labeled *E. coli* ribosomal RNA was used for substrate.

**** ^{3}H-poly rA·oligo dT was used for substrate.

***** ^{3}H-thymidine labeled mouse embryo cell DNA was used for substrate.

Pretreatment of the reverse transcriptase with the inhibitor. Effect of the inhibitor against the reverse transcriptase molecules was examined. The reverse transcriptase was pretreated with the inhibitor. Fig. 2 shows time course of pretreatment of the reverse transcriptase with the inhibitor. Without the inhibitor, activity of the reverse transcriptase was gradually decreased as incubation time was prolonged. This is the

Table 3. Effect of trypsin and proteinase K on the inhibitor

Treatment	30 min.	60 min.
Inhibitor*	440.0**	557.5
Inhibitor + Trypsin	8,236.5	16,172.5
Inhibitor + Proteinase K	4,673.0	11,475.5
Trypsin	20,987.0	23,725.0
Proteinase K	26,755.0	20,515.0
Control	50,700.0	nd***

* 20 units of the inhibitor were used. 20 ug/reaction of trypsin and 50 ug/reaction of proteinase K were used.

** cpm

*** not done

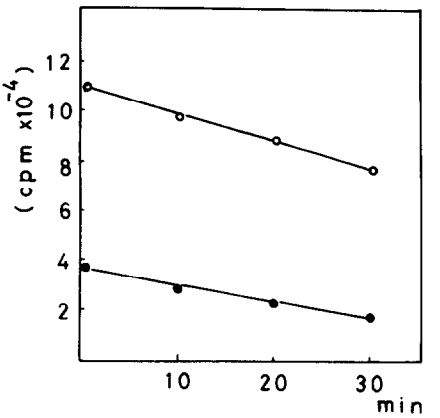

Fig. 2. Pretreatment of reverse transcriptase with the inhibitor. Reverse transcriptase was incubated with the inhibitor at 37°C for 10, 20 and 30 minutes. Then, reaction mixture for reverse transcriptase assay was added. After incubation for 30 minutes, acid insoluble radioactivities were assayed. Without the inhibitor (o——o), with the inhibitor (●——●).

heat inactivation curve of the reverse transcriptase at 37°C. If the inhibitor attacks the reverse transcriptase molecules directly, slope of the curve with the inhibitor is sharper than that of the control. In Fig. 2, the gradient of the control curve is -0.28 (arbitary unit) and that with the inhibitor is -0.17.

It is suggested that the inhibitor does not attack the reverse transcriptase molecule directly. It shows that the inhibitor might not be protease(s). The mechanism of action of the inhibitor against the reverse transcriptase will be reported elswhere. In this paper, we reported that an inhibitor of reverse transcriptase was found in the cytoplasm of the cells. The inhibitor is a protein which has a molecular weight of 85,000. It does not inhibit DNA dependent DNA polymerase of E. coli pol I. Destruction of template-primer or the product of reverse transcription was not observed (data were not shown).

It is considered that the inhibitor might play an important role in retrovirus DNA synthesis in the cytoplasm of the infected cells. It might be one of the mechanisms of resistance of the virus infection. Quantitative assay of the inhibitor of the cells which have different sensitivity of retrovirus should be done. Temin proposed protovirus

hypothesis (6) which featured a central role for reverse transcriptase. According to this concept, information exchange from DNA to RNA and back to DNA was a normal cellular process leading to cell differentiation via modification of gene expression. The inhibitor reported here might play an important role in regulation of these processes. Recently, a specific inhibitor of reverse transcriptase in human placentas was reported (7). It seems to interact with reverse transcriptase directly and be not protein in nature. It might be a different inhibitor from that reported here.

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