Vol. 80, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1978 Pages 729-734

HIGH MOLECULAR WEIGHT VIRAL REVERSE TRANSCRIPTASE IN MOLONEY SARCOMA VIRUS TRANSFORMED CELLS

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Received November 28,1977

SUMMARY: The viral reverse transcriptase in the cytoplasm of Moloney sarcoma virus transformed rat cells was purified by the same methods as used for purification of the reverse transcriptase (EC 2.7.7.7) from the mature virions produced by the same cells. The cytoplasmic reverse transcriptase had a molecular weight of 105,000 and that of the reverse transcriptase from the virions harvested at 20 minutes incubation was mainly 105,000. A small amount of a molecular weight of 70,000 enzyme was included. At 2 hours incubation, the enzyme consisted of the proteins of a moleclar weight of 70,000 and a small amount of a molecular weight of 105,000. At 4 hours incubation, a molecular weight of the viral reverse transcriptase was 70,000. It is suggested that the maturation of the enzyme occurs in the virus particles immediately after budding of the particles from the cell surface.

INTRODUCTION: Several different DNA polymerases are present in mammalian cells. They are DNA polymerase α,β and γ(1). Another enzyme which resembles virion-associated DNA polymerases, has been discovered in acute human leukemic cells (2). In order to examine this enzyme and its role in cancer cells, it is necessary to have knowledge about the viral reverse transcriptase in the virus transformed cells. In this report, isolation and purification of the viral reverse transcriptase was performed with Moloney sarcoma virus transformed rat cells by ion-exchange chromatography and density gradient centrifugation. And time course of transition of the high molecular weight form of the enzyme to the low molecular weight form was examined.

MATERIALS AND METHODS: The rat embryo cells transformed by Moloney sarcoma virus, 78A-1 (3), 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 virus particles in tissue culture fluid were purified by sucrose density gradient centrifugation. The purified viruses were disrupted by Nonidet P-40 (NP-40) treatment and centrifuged at 36,000 rpm for 1 hour. The cells ($2\text{-}4\times10^9$ cells in 4-6 roller bottles) were harvested by trypsinization, washed with phosphate buffered saline four times and suspended in 100 ml of 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM mercaptoethanol. The cells were kept for 20 minutes in an ice bath, broken with a Dounce homogenizer and centrifuged for 10 minutes at 1,000 x g. The supernatant was centrifuged at 10,000 x g for 20 minutes. Then the supernatant was centrifuged at 105,000 x g for 1 hour. NP-40 and glycerin were added to the supernatant at 0.2 and 20 % of final concentration respectively.

0006-291X/78/0804-0729$01.00/0

The disrupted viruses and the cytoplasmic supernatants were applied to DEAE cellulose column, previously equilibrated with 0.01 M Tris-HCl pH 7.4, 50 mM KCl, 10 mM mercaptoethanol, 0.2 % NP-40 and 20 % glycerin. The column was washed with the same buffer and eluted 8 column volumes of a linear gradient from 0.05 to 0.4 M KCl in the same buffer. The reverse transcriptase activity of each fraction from the column was assayed as follows. The reaction mixture contained, in a final volume of 50 µl, 50 mM Tris-HCl pH 8.1, 1 mM manganese acetate, 60 mM KCl, 5 mM dithiothreitol, 0.02 % NP-40, 5 µg/ml of $(dT)_{12-18} \cdot (rA)_n$ (P-L Biochemicals, Inc.,) and 25 µC/ml [^{3}H] dTTP (specific activity 53.7 C/mmole). Incubation was carried out at 37°C for 30 minutes and radioactivity incorporated into an acid-insoluble form was assayed.

The antibody against the viral reverse transcriptase was prepared by injection of purified Moloney sarcoma virus DNA polymerase into rabbits. The IgG fraction was obtained by the method described by Todaro and Gallo (4).

Glycerol gradient centrifugation of the viral reverse transcriptase from the mature virions and the cytoplasmic fraction was done as follows. Five to twenty percent (V/V) glycerol gradients were prepared in 10 mM Tris-HCl pH 7.5, 0.1 M KCl, 10 mM mercaptoethanol and 0.2 % NP-40. Fifty µl of the enzyme solution, 50 µl of bovine serum albumin (10 mg/ml) and 50 µl of human gamma globulin (10 mg/ml) in distilled water and 50 µl of the same buffer were mixed and overlayed onto glycerol gradient columns. They were centrifuged for 17 hours at 45,000 rpm in a Spinco SW 50.1 rotor. Fractions of approximately 0.2 ml were collected dropwise from the bottom of each tube and were assayed for reverse transcriptase activity and amount of proteins.

RESULTS AND DISCUSSION: The cytoplasmic reverse transcriptase activity was eluted as two peaks from the DEAE-cellulose column. The first peak (peak I) was found in the void volume and the second peak (peak II) was eluted at the position of 0.16 M KCl. The peak I consisted of 75-80 % of total activity. The reverse transcriptase from the mature virions was eluted as single peak at the same position to the peak I from the cytoplasm. Each peak was collected separately and further purified with phosphocellulose and hydroxylapatite. The peak I from the cytoplasm and the reverse transcriptase from the mature virions were eluted as single peak from the phosphocellulose column at the position of 0.20-0.21 M and 0.22-0.23 M KCl respectively.

The purified enzymes from the peak I and II of DEAE-cellulose column were tested by neutralization with the antibody against the purified reverse transcriptase from the mature virions. The enzyme from the peak I was clearly inhibited by the immunoglobulin with significant inhibition detected with less than 10 µg of globulin. In contrast, up to 100 µg of globulin from the same rabbit did not significantly inhibit the activity of the peak II from Moloney sarcoma virus transformed cells (Fig. 1). The reverse transcriptase from the mature virions which was eluted at the position of the peak I of the cytoplasm,

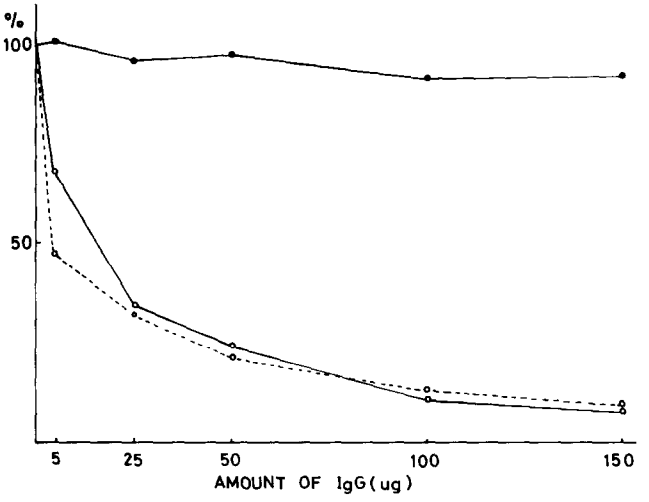

Fig. 1. Inhibition of RNA dependent DNA polymerase from the cytoplasm of the viral transformed cells and the virions by the antibody against the enzyme from the virions. The mixture of the enzyme and gamma globulin was kept for 30 minutes in an ice bath and 60 minutes at room temprature. Then equal volume of reaction mixture for RNA dependent DNA polymerase assay was added. The reaction was carried out for 30 minutes at 37° C. The reverse transcriptase from the virions (o——o), the enzyme from the peak I of DEAE cellulose column chromatography (o·····o), and the enzyme from the peak II of DEAE cellulose column chromatography (●——●).

was also inhibited by that antibody. Thus, the peak I from the cytoplasm was identified as the viral reverse transcriptase. The peak II is supposed to be DNA polymerase γ.

Fig. 2 shows glycerol gradient centrifugation of the reverse transcriptase from the cytoplasm and from the mature virions harvested at 24 hours incubation. Bovine serum albumin and human gamma globulin were used as markers. The enzyme from the virions had a molecular weight approximately 75,000 and the enzyme from the cytoplasm had a molecular weight of 105,000-110,000.

In order to examine time course of molecular weight shift of the viral reverse transcriptase from the cytoplasmic form to the virion form, the virus was harvested at 20 minutes, 2 hours and 4 hours incubation. The enzyme from the virions were centrifuged in glycerol gradient columns for determination

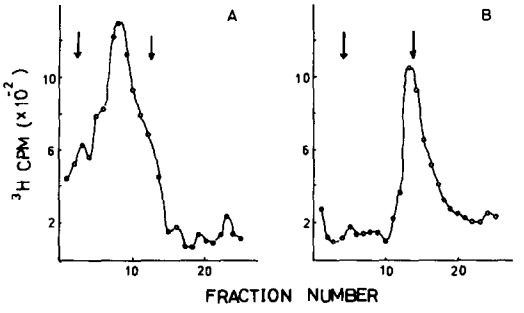

Fig. 2. Glycerol gradient centrifugation of the reverse transcriptase from the cytoplasm and the virions. Five to twenty percent (V/V) glycerol gradient prepared in 10 mM Tris- HCl pH 7.5, 0.1 M KCl, 10 mM mercaptoethanol, and 0.2 % Nonidet P-40, was centrifuged at 45,000 rpm for 17 hours at 4° C in a Spinco SW 50.1 rotor. Fractions of approximately 0.2 ml were collected dropwise from the bottom of the tubes. A) the enzyme from the cytoplasm. B) the enzyme from the virion harvested at 24 hours incubation. Arrows show the position of human gamma globulin (left, molecular weight 160,000) and bovine serum albumin (right, molecular weight 67,000). Left side of the figure is bottom of the gradient column.

of a molecular weight (Fig. 3). The molecular weight of the enzyme from the virions harvested at 20 minutes incubation was 105,000. There was a little shoulder at the position of a molecular weight of 70,000 (Fig. 3, A). Fig. 3, B and C show the glycerol gradient centrifugation patterns of the reverse transcriptase from the virions harvested at 2 and 4 hours incubation respectively. The samples harvested at 2 hours incubation showed two peaks at the position of a molecular weight of 105,000 and 70,000. The samples harvested at 4 hours incubation showed a single peak at the position of a molecular weight of 70,000. The reverse transcriptase from the virions harvested at 24 hours incubation has, also, a molecular weight of 70,000 (data are not shown). It is suggested that the viral reverse transcriptase was present in the cytoplasm as high molecular weight form and became low molecular weight form immediately after maturation of the viruses.

All available evidence indicates that the reverse transcriptase from the mammalian oncorna viruses have a single subunit of 70,000 in molecular weight

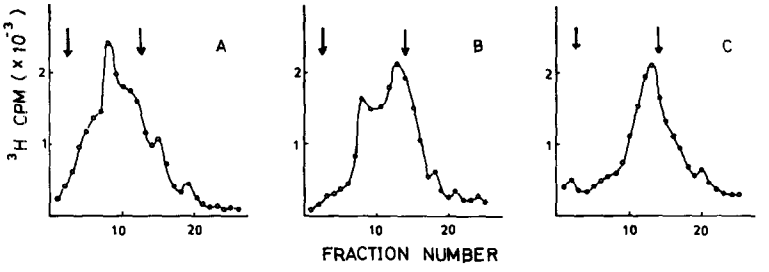

Fig. 3. Glycerol gradient centrifugation of the reverse transcriptase from the virions harvested at different intervals. Centrifugation was performed as described in legend of Fig. 1. A) Reverse transcriptase from the virions harvested every 20 minutes. B) Reverse transcriptase from the virions harvested every 2 hours. C) Reverse transcriptase from the virions harvested every 4 hours. Arrows show the position of bovine serum albumin (right) and human gamma globulin (left). Left side of the figure is bottom of the gradient.

with exception of Mason-Pfizer monkey virus reverse transcriptase which had a molecular weight of 110,000 (5). Mondal et al. reported a high-molecular-weight form of RNA-directed DNA polymerase in the cytoplasmic particles of human leukemia cells and gibbon ape lymphosarcoma cells (6). The enzyme had a molecular weight of 130,000-140,000 which became a low-molecular-weight form by treatment in 0.5 M KCl. It is uncertain that the stoichiometric relationship of two enzyme forms is a monomer dimer relationship. In RD 114 virus produced by human amnion cells, two forms of RNA dependent DNA polymerase were reported (7). The molecular weight of these enzyme was 70,000 and 95,000. The small enzyme is supposed to be identical to the reverse transcriptase of the virus produced by cat cells (RD 114 cells). It is suggested that the large enzyme is a precusor of the small enzyme. It is still not clear that high molecular weight form of the reverse transcriptase of Moloney sarcoma virus corresponds to β form of avian oncorna viruses. Verma speculated temptingly that murine reverse transcriptase is also a two-subunit enzyme, but the larger or precursor subunit is very labile (8). Structure and nature of this enzyme or effect of protease inhibitor to this enzyme will be reported elsewhere.

ACKNOWLEDGEMENT: This work was partially supported by the Ministry of Education, Science and Culture of Japan.

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