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Inhibition of avian myeloblastosis virus reverse transcriptase by an RNA-binding protein from plasma membranes of normal and tumor cells

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Abstract. Purified plasma membranes from normal rat liver, a rat hepatoma and a rat hepatic fibrosarcoma have been shown to contain a protein which drastically inhibits avian myeloblastosis virus reverse transcriptase activity. The inhibition is caused by the binding of the protein to the template. The binding and the consequent inhibition of enzyme activity are template-specific; copying of RNA templates is inhibited whilst that of DNA templates remains unaffected. Investigations using different templates suggest that the inhibitory protein has a stronger binding affinity for G, C-rich templates. The inhibitor appears to have a wide distribution in plasma membranes from diverse sources.

Keywords. Reverse transcriptase; RNA-binding protein; plasma membranes.

Introduction

During the course of a study of the role of host factors in the infection of cells by retroviruses, we have isolated and partially purified a 120,000 dalton protein from plasma membranes of chicken embryonic cells. This protein causes a four-fold stimulation of reverse transcriptase activity (Padhy *et al.,* 1976; Das *et al.,* 1978). We have also reported the presence of an inhibitory activity to reverse transcriptase in plasma membrane preparations from chicken embryonic cells (Das *et al.,* 1978). An examination of plasma membrane preparations from non-embryonic sources such as rat liver, a rat hepatoma (Zajdela ascitic hepatoma, ZAH) and a rat hepatic fibrosarcoma (Yoshida ascitic fibrosarcoma, YAS) has now shown that although no stimulatory factor is present in plasma membranes from these sources, there is a strong inhibitory activity in both normal and tumor plasma membranes. We report here the identification and characterization of this activity, and demonstrate that the inhibition is brought about by the binding of the inhibitor to the RNA template.

Materials and methods

Unlabelled deoxynucleoside triphosphates and synthetic templates were obtained from PL Biochemicals. (Me-^{3}H)-TTP (56 Ci/mmol) and (^{3}H)-dGTP (8 Ci/

Abbreviations used: ZAH, Zajdela ascitic hepatoma; YAS, Yoshida ascitic fibrosarcoma; AMV, avian myeloblastosis virus.

mmol) were obtained from Amersham-Searle, and calf thymus DNA and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Chemicals for enzyme assays were obtained from Patel Chest Institute, New Delhi. All other chemicals were of analytical grade. Purified avian myeloblastosis virus (AMV) reverse transcriptase was a gift from Dr J. W. Beard, Life Sciences Building, St. Petersburg, Florida, USA.

Tumor cells: Zajdela ascitic hepatoma (ZAH) and Yoshida ascitic fibrosarcoma (YAS) cells were maintained by serial transplantation of the tumor in Wistar rats. ZAH cells were obtained from Dr. F. Zajdela, Institut du Radium, Orsay, France and YAS cells were obtained from Cancer Research Institute, Parel, Bombay.

Plasma membrane preparation: Plasma membranes were prepared by three different methods as described elsewhere (Ray, 1970; Aaronson and Touster, 1972; Lesco *et al.,* 1973).

Isolation of AMV-RNA: AMV-RNA was isolated from purified AMV using phenol-cresol extraction as described earlier (Das and Mink, 1979).

Enzyme assays: Reverse transcriptase was assayed as described earlier (Parnaik and Das, 1981). 5′-Nucleotidase (Gurd and Evans, 1974), succinic dehydrogenase (Earl and Korner, 1965), alkaline phosphatase (Pekarthy *et al.,* 1972), alkaline phosphodiesterase I (Pekarthy *et al.,* 1972), inorganic pyrophosphatase (Shatton *et al.,* 1981) and glucose-6-phosphatase (Swason, 1955) were assayed as described.

Protein estimation: Protein concentrations were estimated by a modification of Lowry's method (Lowry *et al.,* 1951). Membrane suspensions (0.25 ml) were added to 1 ml alkaline tartarate solution (0.2% Na, K tartarate, 2% sodium carbonate and 0.002% cupric sulfate in 0.1M NaOH) and incubated at room temperature for 20 min. SDS (0.1 ml, 0.5%), and Folin's reagent (0.1 ml 2N) were then added in quick succession, and the mixture was vortexed. Absorbance at 740 nm was measured after keeping the samples at room temperature for 30 min. Bovine serum albumin solutions of known concentrations were used as standards.

Results

Inhibition of reverse transcriptase

Plasma membranes from normal rat liver, ZAH and YAS cells were isolated and assayed for purity by determining the enrichment of marker enzymes. Plasma membrane markers (5′-nucleotidase, alkaline phosphodiesterase I and alkaline phosphatase) were enriched 10-20 fold, whereas cytoplasmic contaminants (succinic dehydrogenase, inorganic pyrophosphatase and glucose-6-phosphatase) were undetectable. The membrane preparations were solubilized in 1% NP-40 and added to reverse transcriptase reaction mixtures. The final concentration of NP-40 was adjusted to 0.5% in the reaction mixtures. The concentration of membrane preparations ranged from 0.1 to 0.5 mg/ml according to the requirement of the experiments and are shown in the legends to tables and figures for individual experiments. Membrane preparations from normal liver as well as ZAH and YAS cells inhibited the copying of ploy rA.oligo dT by reverse transcriptase (figure 1). The drastic inhibition by liver plasma membranes was

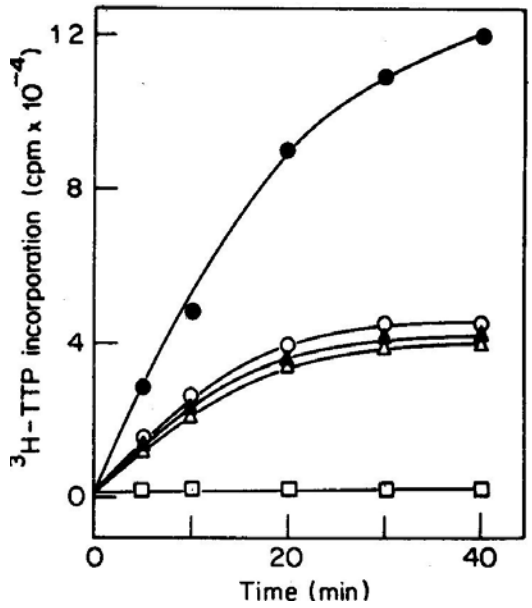

Figure 1. Reaction mixtures containing enzyme (0.72 U), 0.1 ml of solubilised plasma membranes in 1% NP-40 (30 μg of total protein) and components of the assay mixture (with poly rA.Oligo dT as template) in a total volume of 0.2 ml were incubated at 37°C. Aliquots (25 μl) were removed at various time intervals and assayed for reverse transcriptase activity as described in Methods. (●) Enzyme alone (in 1% NP-40); (▲) enzyme and liver plasma membrane; (Δ) enzyme and ZAH plasma membranes; (O) enzyme and YAS plasma membranes;(■)liver plasma membranes and enzyme in presence of 0.02 mM cysteine. ZAH plasma membranes and enzyme in presence of 0.02 mM cysteine showed the same kinetics as ZAH plasma membranes and enzyme in the absence of cysteine.

partly due to high levels of alkaline phosphatase activity in the plasma membrane preparations, as shown later. Due to this interfering inhibitory activity in liver plasma membrane preparations, most of the studies on the mechanism of inhibition were performed with tumor plasma membranes. As shown in figure 2, the inhibition was a linear function of the plasma membrane concentration, indicating that the inhibition 'was specific. The inhibitory activity was non-dialysable, suggesting that it was macromolecular in nature.

In order to determine whether the inhibitor was a protein, the plasma membrane preparations were treated with trypsin, followed by the addition of soybean trypsin inhibitor to inactivate trypsin, and then assayed for inhibition of reverse transcriptase. The inhibitory activity was destroyed by treatment with trypsin, indicating that the inhibitor is a protein (see table 1). Furthermore, inhibitory activity was abolished by incubating the solubilized membranes at 80°C for 5 min.

The possibility that the inhibition could also arise from the presence of any endogenous RNA or DNA in plasma membrane preparations that would competitively bind to the enzyme and inhibit poly rA.oligo dT copying was ruled out in the following manner. Membrane preparations were assayed for their ability to support DNA synthesis by reverse transcriptase by the addition of purified membranes (in 0.5% NP-40) to assay mixtures containing enzyme, all four dNTPs and oligo dT as primer but no other added template. Under these circumstances no

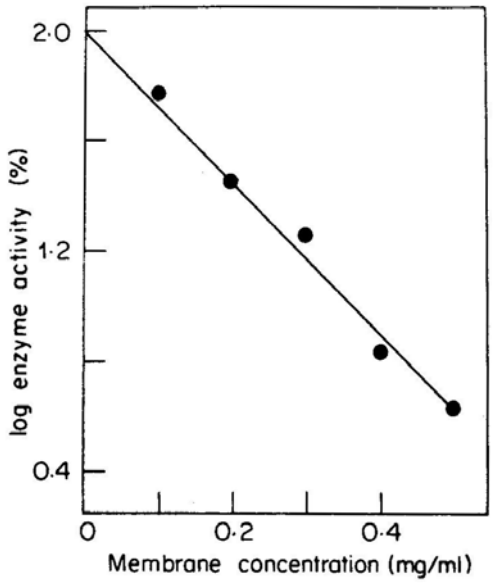

Figure 2. Enzyme samples (0.72 U) were treated with different concentrations of ZAH plasma membranes in 1% NP-40 (0.1 to 0.5 mg/ml) and assayed for activity (with poly rA.oligo dT as template) after 20 min at 37°C as described in Methods.

Table 1. Effect of trypsin treatment on the plasma membrane inhibitor.

Experiment	^{3}H-TTP incorporation (pmol/40 min./0.15 ml)
Standard reaction	318
Reaction with ZAH membranes	95
Reaction with trypsin-treated ZAH membranes	270
Reaction with added trypsin + soybean trypsin inhibitor (mixed prior to addition of enzyme)	305

ZAH plasma membranes (20 µg in 0.5% NP-40) were treated with 1 mg trypsin for 20 min. at 37°C, followed by the addition of 3 mg of soybean trypsin inhibitor. After 20 min. at 37°C, 1.0 U of reverse transcriptase and other components of the assay were added to give a total volume of 0.15 ml, and activity was determined as described in Methods. A control reaction with 0.5% NP-40 in the absence of plasma membranes was carried out in an identical manner. Values for control reactions in the absence of trypsin and SBTI treatment are also indicated. The specific activity of ^{3}H-TTP used was 500 cpm/pmol.

DNA synthesis was evident. The data strongly suggests that the inhibition is specifically brought about by a plasma membrane protein. The inhibitory activity was reproducibly observed from several batches of plasma membrane preparations, isolated by different methods and stored over varying lengths of time.

A series of experiments were carried out to test whether the observed inhibition could arise from the presence of any protease or nuclease activities in the membrane preparations. These were ruled out (results not shown). Protease activity was checked by the Kunitz method (Kunitz, 1947), using standard casein solutions. Ribonuclease and polynucleotide phosphorylase activities were assayed using *Escherichia coli* total RNA as substrate. Any interfering deoxyribonuclease activity in the membrane preparations was ruled out by the absence of degradation of DNA in incubation mixtures containing added DNA and membrane samples (results not shown).

As indicated earlier, liver plasma membranes contained considerable amounts of alkaline phosphatase, whereas tumor plasma membranes contained much lower amounts. In order to determine whether inhibition of reverse transcriptase by the membrane preparations was due to alkaline phosphatase activity, assays were carried out in the presence of 0.02 mM cysteine, a known inhibitor of alkaline phosphatase. Under these conditions, there was no effect on inhibition of reverse transcriptase by tumor membranes. However, the level of inhibition by liver plasma membranes decreased to the level of inhibition by tumor plasma membranes. It is clear from these experiments that whereas there was a genuine inhibition of reverse transcriptase by normal and tumor plasma membranes, the difference in the levels of inhibition by different membranes was due to differences in alkaline phosphatase levels in the membrane preparations. In order to check the presence of other phosphatases in membrane preparations, membranes were incubated with dNTPs. Under these conditions no degradation of dNTPs was observed.

We conclude from these experiments that the inhibition of reverse transcriptase by the membrane preparations is not caused by ribonuclease deoxyribonuclease, protease or phosphatase activities present in the membranes, but is caused by a specific protein inhibitor.

Mechanism of inihibition

The binding of the inhibitor either to the template or the enzyme could result in the observed decrease in DNA synthesis by reverse transcriptase in presence of plasma membrane preparations. If the inhibition is caused by the binding of the inhibitor to the enzyme, then at limiting concentrations of the inhibitor, increase in enzyme concentration should relieve inhibition. However, it was observed that as the enzyme concentration was increased five-fold, the percentage of inhibition did not vary significantly, indicating that the decrease in DNA synthesis was independent of enzyme concentration.

In order to determine whether the inhibitor acts by binding to the nucleic acid template, the effect of increasing template concentration on the extent of inhibition was studied. At limiting concentrations of the inhibitor, an increase in template concentration should relieve inhibition if the inhibitor acts by binding to the template. As shown in figure 3, inhibition is relieved considerably as the template concentration is increased. This observation strongly suggests that the inhibitor acts by binding specifically to the nucleic acid template.

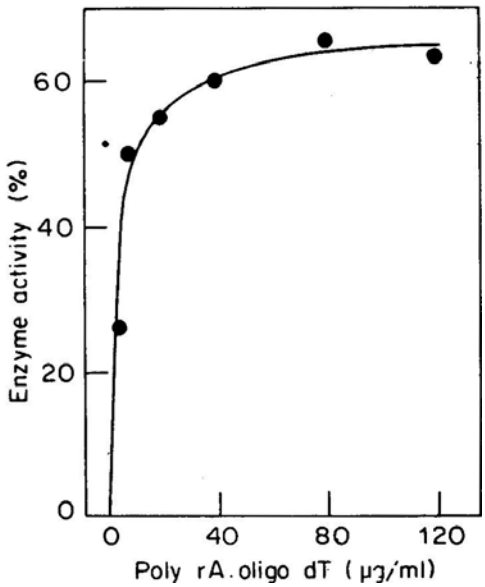

Figure 3. ZAH plasma membranes (10 µg) were added to reaction mixtures containing enzyme (0.24 U), various concentrations of poly rA.oligo dT (4-120 µg/ml) and the rest of the assay components in a volume of 0.1 ml. Enzyme activity was assayed after 10 min. at 37°C as described in Methods. Control reactions in the absence of membranes were carried out in an identical manner at each template concentration.

Template-specificity of inhibitor

The binding of the inhibitor to nucleic acid templates was critically examined using different templates. It can be seen from table 2 that the plasma membrane inhibitor specifically inhibits the copying of RNA templates including native AMV RNA, and has no detectable effect on the copying of two DNA templates: poly dA.oligo

Table 2. Template specificity of inhibitor.

Template	^{3}H-TTP incorporation (pmol/30 min/0.1 ml)		Inhibition %
	−Inhibitor	+Inhibitor	
Poly rA.oligo dT	339.0	97.9	73
Poly rC.oligo dG	3120.0	107.0	97
AMV RNA	9.8	3.0	69
Poly dA.oligo dT	8.0	8.0	0
Activated calf thymus DNA	20.8	19.3	7

Enzyme samples (0.1-2.0 U) were assayed with different templates (20 µg/ml) in the presence of 20 µg ZAH plasma membranes in a total volume of 0.1 ml. Enzyme activity was determined after 30 min. at 37°C as described in Methods. The specific activity of ^{3}H-dNTP (500-5000 cpm/pmol) was varied to give optimum incorporation of radio-activity for the different templates.

dT and activated calf thumus DNA. Furthermore, inhibition is considerably higher with the G, C-containing template, poly rC.oligo dG, suggesting that the inhibitor has a stronger binding affinity for G,C residues.

Experiments were also carried out to check whether the inhibitor specifically affects chain initiation. Rates of DNA synthesis were measured for two sets of parallel assays. In one set, reactions were started by the simultaneous addition of the enzyme and inhibitor to assay mixtures containing all the components of the reaction except the enzyme. In the second set, reactions were allowed to proceed for 5 min by adding aliquots of enzyme alone to the assay mixtures. At this point samples of membrane preparations were added to the reaction mixtures in this set. Both sets of reaction mixtures were incubated at 37°C and the incorporation of labelled TTP was monitored upto 40 min. The results showed no difference in the rates of incorporation of label between the two sets (data not shown). The absence of enhanced inhibition in reactions in which the inhibitor was present at start rules out the possibility of specific inhibition during the chain initiation event.

Discussion

A protein present in plasma membrane preparations from several sources such as normal liver cells, ZAH and YAS cells inhibits RNA-directed DNA synthesis by reverse transcriptase. Our results suggest that a template-inhibitor interaction is responsible for the observed inhibition. The inhibitor interacts specifically with RNA templates, and has a stronger affinity for G, C-containing polynucleotides. Preliminary experiments suggest that the inhibitor is present in membrane preparations from several other sources such as chicken liver cells and neonatal mouse liver cells. Recently, inhibitors of reverse transcriptase have been detected in mouse spleen cells (Rokutanda *et al.,* 1982) and human placental tissue (Nelson *et al.,* 1981). However, the cytoplasmic origin of both inhibitors makes it unlikely that they are similar to the inhibitor we have identified in plasma membranes.

The presence of an RNA-binding protein in plasma membranes from diverse sources raises the question of the functional role of such a molecule. Since replication of the retroviral genome proceeds via an RNA-dependent DNA polymerization, the inhibitory role of an RNA-binding protein could, in principle, provide a protective mechanism for the host. It has also been suggested that RNA-binding proteins might be involved in such diverse phenomena as the induction of interferons by double-stranded RNA molecules (Gordon and Hinks, 1981), modulation of host cell mRNA translation during infection by DNA tumor viruses (Khandjian *et al.,* 1982), compartmentalization of cytoplasmic RNA (DeRobertis *et al.,* 1982) and uptake of RNA by cells (Schell, 1971). It is not known whether these events take place at the cell surface or through the mediation of the plasma membranes. At present we do not know whether the RNA binding protein we have characterized in this study plays a role in any of these cellular events. Purification and further characterization of RNA-binding proteins from plasma membranes are under progress with a view to answer some of these questions.

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