PRESENCE OF VIRAL RNA-INSTRUCTED DNA POLYMERASE IN THE ONCOGENIC SUBVIRAL PARTICLES (VIROSOMES) ISOLATED FROM THE MITOCHONDRIA OF ROUS SARCOMA CELLS

J. KÁRA, M. DVOŘÁK and H. ČERNÁ

Laboratory for Biochemistry of Viral Cancerogenesis, Institute of Experimental Biology and Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

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1. Introduction

Recently we have shown the presence and biosynthesis of virosomes, oncogenic ribonucleoprotein particles in the mitochondria isolated from Rous sarcoma tissue [1, 2]. A high titre of oncogenic viral activity in the Rous sarcoma mitochondria and presence of viral proteins (Gs antigens) in the mitochondria in avian and mammalian tumors induced by Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) have also been demonstrated [3, 4]. These data and further experimental evidence that chloramphenicol and ethidium bromide, the inhibitors of mitochondrial functions, evidently inhibit the RSV replication and malignant transformation of the fibroblasts infected with RSV in culture [2] strongly suggest that the mitochondria play a significant role in the replication of RSV and in the mechanism of malignant cell transformation by this oncogenic virus. Similar conclusions were published by Richert and Hare [5].

In this communication, we are presenting experimental evidence on the presence of Rous sarcoma virus-specific RNA-instructed DNA polymerase, reverse transcriptase [6–11], in the virosomes isolated from the inner membrane and matrix (IM + matrix) fraction of RS mitochondria using the digitonin method of Schnaitman and Greenawalt [13]. Viral DNA polymerase in the isolated virosomes was characterized by inhibition of this enzymatic activity by specific rabbit antiserum, and activation by a synthetic template poly(rA):oligo(dT). Coincidence of reverse transcriptase activity and biological (oncogenic) activity in the virosome fraction isolated by sucrose density gradient centrifugation is also demonstrated.

2. Materials and methods

RS mitochondria and chicken embryo mitochondria were prepared in 0.25 M sucrose [12] from Rous sarcoma tumors induced by SR-RSV and 10-day-old Brown Leghorn chick embryos, respectively. Purified mitochondria were fractionated with digitonin [13] in order to eliminate the possible adsorbtion of RSV. The viral infectivity titre in the IM + matrix fraction was determined by the focal test [14] and presence of Gs viral antigens by the Ouchterlony method [4]. A new method was developed for the isolation of virosomes from the IM + matrix was resuspended in 0.25 M Tris buffer (pH 8.1), containing 0.05 M $MgCl_2$ and 0.25% dithiothreitol (10–20 mg protein per ml) and homogenized by 4 times repeated freezing and thawing. The homogenate was centrifuged in a discontinuous sucrose gradient. Fractions containing virosomes (sedimenting in 58–60% sucrose) were pooled and used for the determination of DNA polymerase activity, as described in the legend to fig. 2, and viral activity [3]. Purified RSV

Table 1
Viral, cell-transforming activity and group-specific antigens present in the inner membrane and matrix fraction of Rous sarcoma mitochondria.

Submitochondrial fraction of RS-mitochondria	Viral activity (FFU/100 μg protein)	Immunoprecipitation in agar (number of precip. zones) using Gs-antisera	
		Hamster anti-Gs	Rabbit anti-Gs
Inner membrane and matrix	1.5×10^4	2	3
Virosomes	4×10^3	1	1

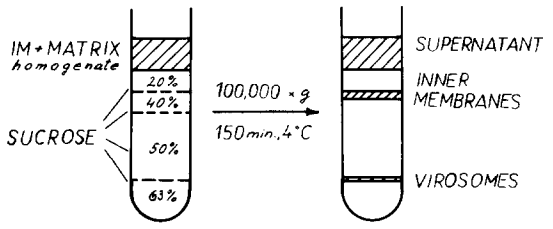

Fig. 1. Discontinuous sucrose density gradient used for the isolation of virosomes from the inner membrane and matrix fraction of Rous sarcoma mitochondria. The gradient was prepared in 5 ml MSE polypropylene centrifuge tube by pipeting 0.75 ml of 63% sterile sucrose solution, overlayered with 1.5 ml 50% sucrose, 0.5 ml 40% sucrose and with 0.5 ml 20% sucrose. All sucrose solutions contained 0.01 M sodium citrate buffer, pH 7.0. Homogenate of IM + matrix fraction (0.5 ml) was layered on the top of the gradient and centrifuged at 100,000 *g* and 4° for 150 min in the MSE 3 × 5 ml swing out rotor. Six drop fractions were collected from the bottom of each tube and sucrose concentration was determined refractometrically.

was prepared as described earlier [3]. Specific rabbit antiserum against RSV DNA polymerase was prepared by repeated immunization of a rabbit with partially purified soluble RSV DNA polymerase [11], by the method described by Aaronson et al. [16]. Protein concentration was determined by the method of Lowry et al. [18]. Poly(rA) (Calbiochem) was used for the preparation of the hybrid poly(rA): oligo(dT). Oligonucleotides (dT) were synthesized using the methods of Khorana and Vizsolyi [19] and Narang et al. [20].

3. Results

As shown in table 1, a relatively high titre of oncogenic viral activity was reproducibly found in the IM + matrix fraction of RS mitochondria. When the preparation of this fraction was homogenized and analyzed in a discontinuous sucrose gradient, an opalescent band of nucleoprotein particles (virosomes) in the interface of 63% and 50% sucrose was visible (fig. 1). By this convenient method the virosomes were separated and concentrated in a small volume (0.4–0.6 ml) in the 60% sucrose solution with the density 1.28 g/cm^3. The virosome fraction contains a significant titre of viral oncogenic activity (table 1) and viral proteins (Gs antigens), as determined by immunoprecipitation (table 1). These results demonstrate the presence of oncogenic subviral particles in the IM + matrix fraction, i.e. inside the RS mitochondria. The technique used practically excludes the possibility that viral infectivity found in the mitochondria is due to Rous sarcoma virions adsorbed to the surface of RS mitochondria.

3.1. *DNA-dependent and RNA-dependent DNA polymerase activities in the virosomes isolated from RS-mitochondria*

The fraction of virosomes sedimenting at the density 1.28 g/cm^3 in the sucrose gradient was used immediately for the determination of DNA polymerase activity using denatured calf thymus DNA as template. As shown in fig. 2, there was a significant DNA polymerase activity in the virosomes. The enzyme activity was inhibited by specific rabbit antiserum, whereas normal rabbit serum at the same protein concentration did not affect the enzymatic reaction (fig. 2).

Pretreatment of the virosomes with 0.1% Nonidet P40 (at 0° for 60 min) did not increase DNA polymerase activity, indicating that the preparation of virosomes does not contain intact virions, but subviral ribonucleoprotein particles [1] characterized by sedimentation at a higher density than RSV, and free of lipoprotein envelope. DNA polymerase activity from RSV was inhibited by 50% in a similar manner as virosomal DNA polymerase in the presence

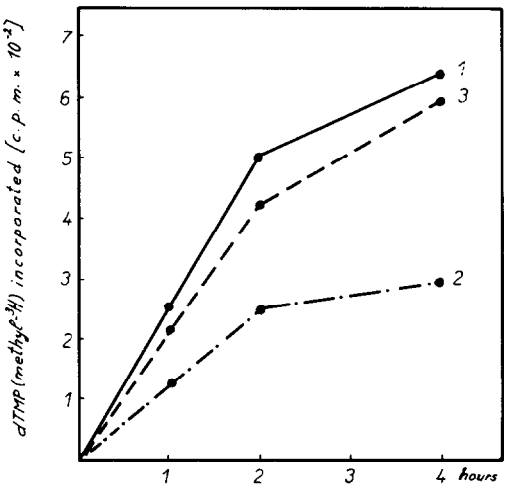

Fig. 2. Inhibition of virosomal DNA polymerase by specific rabbit antiserum. Curve 1: Time course of incorporation of dTTP-methyl^{3}H into acid-insoluble product, in the absence of antiserum. Curve 2: Time course of virosomal DNA polymerase activity in the presence of rabbit anti-RSV DNA polymerase antiserum (500 μg protein). Curve 3: Virosomal DNA polymerase activity in the presence of normal, inactivated rabbit serum (500 μg protein). The reaction mixture (final volume 0.25 ml) contained the following components: 0.1 M Tris buffer (pH 8.1), 0.08 M KCl, 0.01 M $MgCl_2$, 5 mM dithiothreitol, 1 μCi dTTP (methyl-^{3}H) (Amersham, specific radioactivity 10 Ci/mmole), dATP, dGTP and dCTP (20 nmoles each), 15 μg of heat-denatured calf-thymus DNA and 50 μl of virosome fraction (30 μg protein). The mixture was incubated at 38° for several hours, as indicated in the figure. The reaction was stopped by addition of 3 ml 10% trichloroacetic acid (ice-cold) and acid-insoluble precipitate was filtered and washed on Schleicher and Schüll BA 85 membrane filters. The radioactivity on the dried filters was measured as described previously [1]. The radioactivity in the "blank" sample (precipitated at time 0 without incubation) was subtracted.

of specific rabbit antiserum (500 μg of protein) as shown in fig. 3.

3.2. *Detection of the RNA-dependent DNA polymerase activity in virosomes*

Results presented in table 2 show that the virosomal DNA polymerase is activated by single-stranded DNA (denatured calf-thymus DNA) and by poly(rA): oligo(dT) template, thus exhibiting a similar template dependence as the reverse transcriptase of RSV. These results demonstrate that virosomes, synthesized

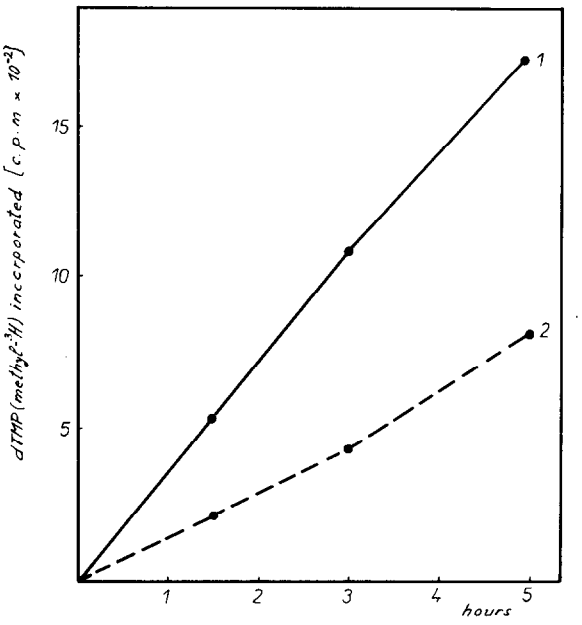

Fig. 3. Inhibition of RSV-DNA polymerase by specific rabbit antiserum. Curve 1: Viral DNA polymerase activity in the absence of serum. Curve 2: Viral DNA polymerase activity in the presence of specific rabbit antiserum (500 μg protein). The composition of the reaction mixture is as described in fig. 2. RSV preparation, treated with 0.1% Nonidet P40 at 0° for 60 min (100 μg protein per assay) was used as enzyme.

in the inner compartment of RS mitochondria [1], do contain a virus specific RNA-dependent and DNA-dependent DNA polymerase.

4. Discussion

Data presented in this communication confirm and extend our earlier findings [1–4] and provide a direct experimental evidence that subviral, oncogenic ribonucleoprotein particles (virosomes) containing viral RNA-dependent DNA polymerase activity are located in the IM + matrix fraction of RS-mitochondria. Removal of the outer mitochondrial membrane of RS-mitochondria by the digitonin fractionation excludes practically the possibility of contamination of the IM + matrix fraction by RSV adsorbed eventually to the surface of the mitochondria. A relatively high titre of the oncogenic virosomes present in the

Table 2
Reverse transcriptase activity in virosomes and in the lysate of Rous sarcoma virions (with 0.1% Nonidet P-40) in the presence of various templates.

Template	DNA polymerase activity (dTMP-met-^{3}H incorporated, cpm)			
	in virosomes		in RSV lysate	
	a	b	a	b
Endogenous	180	840	212	905
Denat. calf-thymus DNA, 50 μg	995	1,809	1,251	1,893
Poly (rA), 50 μg	–		561	
Poly (rA).oligo (dT), 25 μg	–	–	1,568	6,711
Poly (rA).oligo (dT), 50 μg	1,023	1,441	2,340	

The composition of the reaction mixture as described in fig. 2, with the exception that in some cases DNA template (single stranded, denatured calf-thymus DNA) was substituted with poly (rA)-oligo $(dT)_{4-10}$ or poly (rA), as indicated in the table. In the DNA polymerase assay with the virosome preparation two concentrations of virosomal proteins were used: a) 30 μg protein per assay, b) 55 μg protein per assay and the concentration of dTTP-met-^{3}H was 4 μCi per assay. In the DNA polymerase assay in RSV lysate two concentrations of viral proteins were used: a) 200 μg protein per assay, b) 450 μg protein per assay. The enzymatic assay was carried out for 3 hr at 39°.

IM + matrix fraction (table 2) and incorporation of [^{14}C] amino acids and [^{3}H] uridine into the virosomes during *in vitro* labelling of the isolated RS-mitochondria [1, 2] strongly suggest that these particles are synthetized in the mitochondria of Rous sarcoma cells and probably represent an intermediate stage in the development of RSV. The inhibition of RSV replication and cell transformation by ethidium bromide and chloramphenicol [2, 5] support indirectly the conclusion that mitochondria play an important role in the replication of RSV in Rous sarcoma cells.

Reverse transcriptase present in the virosomes exhibits a similar template specificity characteristic of RNA-dependent DNA polymerase of C-type oncogenic RNA viruses [21, 22]. Moreover, DNA polymerase in the virosomes is inhibited by the specific rabbit antiserum prepared against RSV reverse transcriptase [16, 17]. These characteristics as well as physical separation of virosomes from the inner membrane of RS mitochondria distinguish the virosomal reverse transcriptase from DNA-directed mitochondrial polymerase located in the inner mitochondrial membrane [23, 24]. Recently, an RNA-dependent DNA polymerase activated by Mn^{2+} ions has been isolated from chick embryo cells [25, 26]. An RNA-dependent DNA polymerase in the rat liver and cerebral cortex mitochondria [27] has also been reported. At present we do not know if a similar enzyme is present in the normal chick embryo mitochondria.

Purification of virosomal DNA polymerase and a more detailed characterization of this enzyme is in progress.

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