A POSSIBLE BIOLOGICAL FUNCTION OF THE PROTEIN KINASE ASSOCIATED WITH VACCINIA AND VESICULAR STOMATITIS VIRIONS

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Received 18 January 1974

1. Introduction

Protein kinase which transfers the γ-phosphoryl group of ATP and dATP to *O*-seryl and *O*-threonyl linkage in proteins has been detected in purified virions of several enveloped viruses; i.e., vaccinia virus [1], vesicular stomatitis virus (VSV) [2], herpes viruses [3, 4], frog polyhedral cytoplasmic deoxyribovirus (PCDV) [5, 6], influenza, parainfluenza viruses [7], and *RNA* tumor viruses [2, 7]. The virion-associated protein kinase was activated by disruption of the envelope by treatment with nonionic detergent and dithiothreitol (DTT) [1–7]. Upon incubation of the activated virions in vitro with [γ-^{32}P] ATP, certain species of the viral proteins of these viruses were phosphorylated to various degrees [1, 2, 4, 6]. Although the virion-associated protein kinase appeared essential for the infectivity of PCDV [5], its function in viral replication is unknown with any of these viruses.

In view of the fact that the viruses which contain nucleic acid polymerase as an integral component of viral core also carry protein kinase (with an exception of reovirus, unpublished data), we have developed an experimental system to examine whether these two enzymes are functionally related in the virions. In this communication we present evidence that the phosphorylation of viral proteins may be involved in the activation of viral genome transcription in vaccinia virus and VSV.

2. Materials and methods

2.1. *Viruses*

Vaccinia virus (strain WR) was grown in suspension cultures of L-cells and purified by the procedure of Paoletti and Moss [1]. VSV (Indiana serotype) was grown in monolayer cultures of BHK-21 cells, and purified as described by Aaslestad et al. [8].

2.2. *Assay for enzymes*

Virion-associated RNA polymerase and protein kinase were assayed by determining the incorporation of ^{3}H of [5–^{3}H] UTP and ^{32}P of [γ-^{32}P] ATP into acid-insoluble materials, respectively. The virus preparations were preincubated in a 0.05 M Tris–Cl$^-$ buffer (pH 8.0) containing 0.2% of Nonidet P-40 (Np-40) and 0.04 M of DTT at 20° C for over 10 min then diluted to make a final reaction mixture (a total volume of 0.2 ml). Composition of the reaction mixture is described in the individual legend. Incubation was at 37° C for vaccinia virus and *E. coli* enzyme and at 31° C for VSV. The reaction was terminated by adding 200 μg of yeast RNA and 2 ml of 10% trichloroacetic acid (TCA) containing 0.02 M NaPP. The precipitate formed was collected by centrifugation, dissolved in 1 ml of 0.1 M Tris–Cl$^-$ (pH 8.0) containing 1 mM EDTA, and again precipitated by adding 2 drops of 70% TCA. The precipitate was

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collected on a millipore membrane filter and washed with 5% TCA. The filter was dried and the radioactivity determined in a liquid scintillation counter.

2.3. *Chemicals*

Radioactive materials were purchased from New England Nuclear Corp., Boston. Unlabeled nucleotide triphosphates were from Calbiochem, Los Angeles, and β, γ-methylene-ATP (or AMPPCP) and *E. coli* RNA polymerase were from Miles Laboratories, Inc., Kankakee.

3. Results and discussion

3.1. *Reaction kinetics of the virion-associated protein kinase and RNA polymerase*

Incubation of vaccinia virus or VSV in a reaction mixture containing [γ-^{32}P] ATP and [^{3}H] UTP (in the presence of unlabeled GTP and CTP) resulted in the incorporation of both ^{32}P and ^{3}H into acid-insoluble materials (fig. 1). The incorporation of ^{3}H represented viral messenger RNA synthesis mediated by the virion-associated RNA polymerase [8–10], while most part of the ^{32}P incorporated, if not all, appeared to represent phosphorylation of viral proteins. In agreement with other workers (1–4, 7], the incorporation of ^{32}P was unaffected by the omission of CTP, UTP and GTP, and the ^{32}P incorporated was resistant to digestion with pancreatic ribonuclease in a 0.01 M NaCl solution but rendered acid-soluble after digestion with pronase (data not shown). Furthermore, the incorporation of ^{32}P was markedly reduced by the addition of unlabeled dATP, and the reduction was proportional to the ratios of [γ-^{32}P] ATP to unlabeled dATP added (table 1). The result suggested that, like with other viruses [7], dATP was as good a substrate for the protein kinase of vaccinia and VSV as ATP and competed with [γ-^{32}P] ATP for the enzyme. This was a further indication that the incorporation of ^{32}P unrelated to the synthesis of RNA. The result shown in fig. 1 therefore indicated that the phosphorylation of viral proteins took place during the in vitro RNA synthesis and that the phosphorylation reached a plateau before the RNA synthesis took place at a maximum rate.

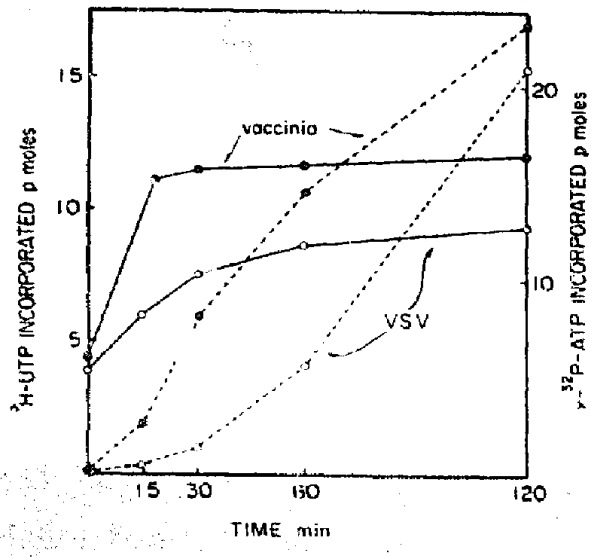

Fig. 1. Reaction kinetics of the RNA polymerase and protein kinase associated with vaccinia and VSV virions. The reaction mixture (0.2 ml) contained 3 nmoles (50 μCi) of [5-^{3}H] UTP, 0.01 μmoles (0.25 μCi) of [γ-^{32}P] ATP, 0.1 μmoles each of unlabeled GTP and CTP, 2 μmoles of $MgCl_2$, 10 μmoles of Tris–Cl⁻ (pH 8.0), 10 μmoles of KCl, 4 μmoles of DTT, 0.02% (vol./vol.) of Nonidet P-40 and 20 μg of the purified vaccinia or 50 μg of VSV virions. Solid lines represent the incorporation of ^{32}P and broken lines that of ^{3}H. Open circles represent the incorporation for VSV and closed one for vaccinia virus.

Table 1
Reduction of ^{32}P incorporation in the presence of unlabeled dATP

[γ-^{32}P] ATP (mM)	dATP (mM)	Relative level of ^{32}P incorporation (%)	
		VSV	Vaccinia v.
0.05	0	100*	100*
0.05	0.05	48.8	47.1
0.05	0.50	12.7	16.2
0.05	5.00	0.6	0.0

The reaction conditions were the same as those described in the legend to fig. 1 except that [5-^{3}H] UTP, unlabeled CTP and GTP were omitted. Unlabeled dATP was added as indicated and the reaction mixture was incubated for 30 min.

* The amounts of ^{32}P incorporated in the absence of dATP were 5.6 and 8.2 pmoles (0-time control values were subtracted) for VSV and vaccinia virus, respectively. Values are the mean of duplicated assay.

3.2. *Requirement for a phosphate donor in the viral genome transcription*

The above results suggested that the viral genome transcription might be stimulated (or initiated) by the preceding phosphorylation of viral proteins. To test this hypothesis we have attempted to carry out RNA polymerase reaction with or without phosphate donor for protein kinase. This was accomplished by using an ATP analogue in which the oxygen atom between the β- and γ-phosphorous atoms replaced by $-CH_2-$; i.e., β,γ-methylene-ATP or AMPPCP. This analogue will not serve as substrate for protein kinase reaction that requires cleavage of β—γ bond while it does substitute for ATP in the RNA synthesis with *E. coli* RNA polymerase (table 2). To the contrary, dATP serves as substrate for protein kinase but not for RNA polymerase. It was therefore possible to use these two ATP analogues to determine whether protein kinase activity is necessary for viral RNA polymerase to be active. If viral RNA synthesis is initiated by the phosphorylation of viral proteins, then the RNA synthesis using AMPPCP should be observed only when dATP is also present in the reaction mixture.

Table 2 summarizes the experimental results obtained with *E. coli* RNA polymerase (calf thymus DNA as template), vaccinia virus and VSV. *E. coli* RNA polymerase utilized AMPPCP as substitute for ATP though high concentrations of the analogue were required to attain a substantial level of RNA synthesis. As expected in this case, the synthesis of RNA with AMPPCP was little affected by the presence of dATP. In contrast, when viral genome transcription was determined, AMPPCP could not substitute for ATP and no significant RNA synthesis was observed with the analogue. A significant level of RNA synthesis was observed only when both AMPPCP and dATP were present. Although the reaction rate with AMPPCP and dATP was considerably slower than that in the complete system, stimulation of the RNA synthesis by the addition of dATP was undoubtedly meaningful. The slow reaction rate with AMPPCP was apparently due to reduced affinity of AMPPCP for RNA polymerases. As shown in table 3, the RNA synthesis using AMPPCP was stimulated with the increasing concentration of the analogue and a significant level of RNA synthesis was observed only at the concentrations over 0.5 mM (while an optimum concentration for ATP was around 0.5 mM under these reaction conditions).

We interpret the results to mean that a phosphate donor (in a form of ATP or dATP) is required for viral RNA polymerase to function in the vaccinia and VSV virions. It is most conceivable that the phosphate donor was utilized as substrate for viral protein kinase

Table 2
Substrate activity of AMPPCP for viral and *E. coli* RNA polymerases and the effect of dATP

Reaction system	^{3}H incorporated		
	VSV (pmoles/4 hr)	Vaccinia v. (pmoles/hr)	*E. coli* RNA-polymerase (pmoles/0.5 hr)
Complete	15	71	3.73
−ATP	0.03	0.01	0.01
−ATP, +dATP	0.06	0.03	0.01
−ATP, +AMPPCP	0.07	0.03	1.04
−ATP, +AMPPCP, +dATP	0.40	0.44	0.80

The complete reaction mixture for the virion-associated enzyme contained in a total volume of 0.2 ml: 3 nmoles (50 μCi) of [5-^{3}H] UTP, 0.1 μmoles each of unlabeled ATP, GTP and CTP, 2 μmoles of $MgCl_2$, 10 μmoles of Tris–Cl^- (pH 8.0), 10 μmoles of KCl, 4 μmoles of DTT, 0.02% (vol./vol.) of NP-40 and 20 μg of the purified virions. When ATP was replaced by its analogues, 1.0 μmole of AMPPCP and 2.5 μmoles of unlabeled dATP were used. For *E. coli* RNA polymerase NP-40 was omitted and 17 μg of calf thymus DNA was added. The values are the mean of duplicated assay.

Table 3
Effect of the concentration of AMPPCP in the viral RNA polymerase reaction

AMPPCP (mM)	^{3}H incorporated (pmoles/hr)	
	VSV	Vaccinia v.
0	0.01	0.01
0.1	0.04	0.01
0.5	0.06	0.09
1.0	0.09	0.14
5.0	0.16	0.29

The reaction conditions were the same as those in the legend to table 2 except that ATP was replaced by dATP (2.5 μmoles) and AMPPCP (the concentration as indicated).

and that the phosphorylation of viral proteins led to the activation of the transcription of viral genome mediated by viral RNA polymerase. Further experiment will disclose what species of viral proteins must be phosphorylated for the activation.

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