Nucleoside Kinase Activities in Tissues Infected with Rous Sarcoma Virus

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Summary

Infection of the chorioallantoic membrane (CAM) of the chick embryo with Rous sarcoma virus (RSV) results in a 10-fold increase in uridine kinase activity of cell-free extracts prepared from the resulting tumor tissue. This enhancement of enzymatic activity is 1st evident 4 days after infection, when pocks are 1st noticed on the CAM. Maximal enzymatic activity is reached 6 or 7 days after infection, when there is a confluent tumor on the CAM. Uridine kinase activity is increased by infection with either the Bryan or the Schmidt-Ruppin strain of RSV.

RSV-induced tumors fail to exhibit a general increase in nucleoside kinase activities. Besides uridine, only cytidine and the analog 6-azauridine of several ribonucleosides and deoxyribonucleosides tested are phosphorylated to a much greater degree in infected CAM than in uninfected CAM.

The increase in phosphorylation of uridine in RSV-infected tissues is not a result of differential rates of catabolism of the substrate, uridine, or the product of the uridine kinase reaction, uridine-5'-phosphate (UMP), since activities of uridine phosphorylase and uracil ribonucleotide phosphatase are similar in control and infected CAM.

Introduction

The biochemical events associated with the replication of RSV in infected tissues are little known. Antimetabolite studies (2, 6, 7, 24) have suggested a requirement for DNA-dependent RNA synthesis following infection with RSV for viral proliferation. Induced or enhanced thymidine kinase activity has been observed in animal cells infected with some DNA-containing viruses (8, 11, 12, 14, 16, 17, 21), whereas reduced uridine kinase activity has been observed in L cells infected with the DNA virus vaccinia (13). As part of a study of nucleic acid synthesis in RSV-infected tissues, we are reporting on the activities of the nucleoside kinases. Besides uridine, only cytidine and the analog 6-azauridine of several ribonucleosides and deoxyribonucleosides tested are phosphorylated to a much greater degree in infected CAM than in uninfected CAM.

The labeled nucleotide formed in the enzymatic reaction was separated from the unchanged nucleoside by the use of DEAE-cellulose ion-exchange paper disks (Whatman diethylaminoethyl cellulose DE-81) (5). Twenty-μl samples of the protein-free reaction mixture were pipetted onto numbered disks, 23 mm in diameter. The disks were washed 3 times with 0.001 N ammonium formate, once with water, and once with ethanol. This procedure removed 97–99% of the uridine-2-14C, and all of the nucleotides were adsorbed onto the ion-exchange disks. The disks were dried and immersed in 5 ml of a solution containing 4 gm of 2,5-diphenyloxazole and 0.05 gm of p-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene and were counted in a Nuclear-Chicago liquid scintillation counter. The results ob-

1 Aided by grants from the National Cancer Institute, C-3811, and the Atomic Energy Commission, AT (30-1)-910.

2 The abbreviations used are: CAM, chorioallantoic membrane; RSV, Rous sarcoma virus; UMP, uridine-5'-phosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; and EDTA, ethylenediaminetetraacetic acid.

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tained by this rapid method were in agreement with the technic of paper electrophoresis and paper chromatography that were used in the earlier stages of this investigation. The assay methods used did not resolve uridine monophosphate, diphosphate, or triphosphate. In the experiments reported below, uridine kinase signifies the phosphorylation of uridine by ATP to uridine monophosphate, diphosphate, and triphosphate. Enzyme activity is expressed in terms of mmoles of nucleoside phosphorylated/mg of protein in 30 min, under the conditions noted above.

PROPERTIES OF URIDINE KINASE. The quantity of uridine phosphorylated was linear with respect to time and enzyme concentration. Maximal activity occurred in the presence of ATP and Mg++. Enzymatic activity. This reached a peak at the 6th or 7th day following inoculation of the virus and then declined. The time and extent of this decline has varied.

URIDINE PHOSPHORYLASE ASSAY. This assay is based on the phosphorolytic cleavage of uridine-2-14C to uracil-2-14C (18, 23). For optimal phosphorylase conditions, the reaction mixture (0.5 ml) contained 8 mmoles of uridine-2-14C (2 X 10^6 cpm); 100 μmoles of phosphate buffer (pH 7.4); and enzyme extract (0.5-1.5 mg). The reaction was incubated at 37°C for 30 min. Under these conditions uracil formation was a linear function of protein concentration. Maximal activity occurred in the presence of ATP and Mg++. Enzyme activity is expressed by the % of 14C activity washed off the DE-81 disks by 0.001 M phosphate (UMP). Catabolism of UMP to uridine was measured by the % of 14C activity washed off the DE-81 disks by 0.001 M ammonium formate.

For optimal phosphorylase conditions, the reaction mixture (0.5 ml) contained 5 mmoles of UMP-2-14C, 50 μmoles of Tris buffer, pH 7.4, and enzyme extract (0.5-1.5 mg). The reaction was incubated at 37°C for 30 min. The amount of UMP cleaved was linear with respect to protein concentration under these conditions.

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The influence of uracil ribonucleotide phosphatase on the uridine kinase reaction was determined by substituting UMP-2-14C for uridine in the uridine kinase reaction mixture and by measuring the catabolism of UMP under these conditions.

Results

ENHANCEMENT OF URIDINE KINASE ACTIVITY. The enhancement of uridine kinase activity 1st became evident at the 3rd or 4th day following inoculation of the CAM with RSV. At this time the beginning of discrete raised pocks appeared on the surface of the infected CAM. As these developed into larger tumors and into a confluent tumor, there was further increase in enzymatic activity. This reached a peak at the 6th or 7th day and then declined. The time and extent of this decline has varied. This variation may be due to different degrees of necrosis in the preparations. An experiment demonstrating the correlation between time of inoculation of the virus and increase in enzymatic activity in the extracts of the infected CAM is shown in Chart 1. Activities of other nucleoside kinases. To determine whether infection with RSV resulted in a general increase in other nucleoside kinase activities, several ribonucleosides and deoxyribonucleosides were tested as substrates for the corresponding nucleoside kinases. Extracts of the CAM infected for 7 days with RSV and the control CAM of the same age were used as controls.

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In Chart 1. Enhancement of uridine kinase activity after infection of CAM of chick embryo with RSV. The assay system (0.5 ml) contained 50 μmoles of Tris buffer (pH 7.4); 16 μmoles of ATP; 10 μmoles of MgCl2, 8 mmoles of uridine-2-14C (2 X 10^6 cpm); and enzyme extract (1.0 mg). Incubation was at 37°C for 30 min. Control CAM, •—•; infected CAM, ○—○.

TABLE 1

Comparison of Ribonucleoside and Deoxyribonucleoside Kinase Activities in Extracts of Infected and Control Membranes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nucleoside Kinase Activities (RSV infected/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>7.6</td>
</tr>
<tr>
<td>6-Aza-uridine</td>
<td>7.7</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1.2</td>
</tr>
<tr>
<td>Cytidine</td>
<td>7.5</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1.0</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.7</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>1.4</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>0.7</td>
</tr>
<tr>
<td>Deoxyeytidine</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a Ratio of mmoles of ribonucleoside or deoxyribonucleoside phosphorylated/mg of protein. RSV, Rous sarcoma virus. The complete assay system is as described in Chart 1. 4H- or 14C-labeled substrates (2 X 10^6 cpm) were used.

To convert a given increase in other nucleoside kinase activities, several ribonucleosides and deoxyribonucleosides were tested as substrates for the corresponding nucleoside kinases. Extracts of the CAM infected for 7 days with RSV and the control CAM of the same age were used as controls. 6-Aza-uridine also served as a substrate, and enhanced conversion occurred to the same degree as with uridine. The rate of phosphorylation of thymidine as well as of the other deoxyribonucleosides by the infected CAM increased slightly over the low activities in control extracts. When thymi-
TABLE 2

UTILIZATION OF URIDINE-2-14C IN EXTRACTS OF CAM* INFECTED WITH ROUS SARCOMA VIRUS

<table>
<thead>
<tr>
<th>Distribution of 14C after 30 min incubation (%)</th>
<th>Uridine</th>
<th>Uridine nucleotides</th>
<th>Uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Standard conditions for kinase assay4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAM-control</td>
<td>90</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>CAM-infected</td>
<td>63</td>
<td>34</td>
<td>3</td>
</tr>
<tr>
<td>B. Conditions altered to favor uridine phosphorylase reaction4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAM-control</td>
<td>46</td>
<td>2</td>
<td>52</td>
</tr>
<tr>
<td>CAM-infected</td>
<td>40</td>
<td>2</td>
<td>58</td>
</tr>
</tbody>
</table>

* The abbreviations used are: CAM, chorioallantoic membrane; and ATP, adenosine triphosphate.

5 Standard uridine kinase assay conditions as described in Chart 1; initial mmole uridine: 8.0.

6 The assay conditions were as follows: 0.5 ml contained 8 mmole of uridine-2-14C (2 X 10^6 cpmp); 100 μmole of orthophosphate buffer (pH 7.4); and enzyme (1.5 mg dialyzed extract). Incubation was at 37° for 30 min. (B differs from A by omission of ATP and Mg++) and addition of orthophosphate.

uridine kinase was assayed daily, there was an increase of less than 50%; activity from the 1st day of RSV infection through the 8th day. The purine nucleosides adenosine and guanosine were converted to about the same degree in the normal and infected CAM.

enzyme enhancement with another strain of RSV. To test the specificity of the Bryan strain of RSV as an enhancing agent of uridine kinase, extracts of tumors from the CAM infected with the Schmidt-Ruppin strain (1) for 7 days were prepared. Essentially identical results were obtained with this strain. Uridine and cytidine were phosphorylated at a rate 7- to 12-fold greater in the infected CAM, whereas thymidine kinase activity increased by only 70%.

Since the observed increase in uridine kinase activity in the infected CAM could have been due to factors other than increased enzyme synthesis, enzyme activities associated with catabolism of both substrate and product were measured in extracts of the CAM infected with the Bryan strain of RSV for 7 days. The possibility of the presence of inhibitors in the control CAM was also studied.

substrate availability. To check that this enhanced enzyme activity is not simply due to differences in the availability of substrate, the catabolism of uridine to uracil by uridine phosphorlyase in the CAM extracts was measured (Table 2). Under standard uridine kinase assay conditions, very little of the substrate is cleaved to uracil. Uridine phosphorlyase activity could be demonstrated by altering conditions to favor formation of uracil: removal of Mg++ and ATP and addition of orthophosphate. Little difference could be demonstrated in the uridine phosphorlyase activity of extracts from normal and infected membranes.

breakdown of UMP. To check that the observed increase in the conversion of uridine to UMP in extracts of the infected CAM did not merely reflect a difference in UMP catabolism between the infected and control CAM extracts, the activity of uracil ribonucleotide phosphatase was measured. The data in Table 3 illustrate that both preparations degraded UMP at similar rates under conditions of the uridine kinase assay. When conditions were altered to favor phosphatase activity by omitting ATP and Mg++, the rates of uridine formation from UMP were similar in both extracts.

presence of inhibitors. The presence of an inhibitor in the control extracts was ruled out by mixing control and infected extracts and obtaining additive results. Combining an extract taken during the early period of infection with an extract from a later phase again yielded additive results.

Discussion

This investigation has demonstrated that there is approximately a 10-fold increase in uridine kinase levels in the CAM infected with RSV (both Bryan and Schmidt-Ruppin strains). These observations are in accord with the communication of Rada and Gregusová (20), who noted enhanced phosphorylation of 6-azauridine in chicken breast muscle tumors and chick embryo cells infected with RSV (Prague strain).

The present observation extends these studies in CAM and investigates other ribonucleoside- and deoxyribonucleoside kinase activities. Our finding that greatly enhanced nucleoside kinase activities were limited to only uridine and cytidine and not to thymidine or deoxycytidine is of particular interest, since this would tend to rule out the possibility of enzymatic differences merely reflecting differences in rates of synthesis between rapidly growing tissue and normal CAM. Studies with rapidly growing tissue, such as regenerating liver (3, 4), have demonstrated a 10- to 25-fold increase in thymidine kinase levels. Skold (23) compared enzyme levels in regenerating liver and noted that deoxycytidine kinase activity increased at a rate

TABLE 3

URACIL RIBONUCLEOTIDE PHOSPHATASE ACTIVITY IN CAM* EXTRACTS

<table>
<thead>
<tr>
<th>Formation of uridine-2-14C from UMP-2-14C after 30 min incubation (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Standard conditions for kinase assay4</td>
<td>36</td>
</tr>
<tr>
<td>CAM-control</td>
<td>30</td>
</tr>
<tr>
<td>CAM-infected</td>
<td>86</td>
</tr>
<tr>
<td>B. Conditions altered to favor uracil nucleotide phosphatase reaction4</td>
<td>74</td>
</tr>
<tr>
<td>CAM-control</td>
<td>86</td>
</tr>
<tr>
<td>CAM-infected</td>
<td>74</td>
</tr>
</tbody>
</table>

* The abbreviations used are: CAM, chorioallantoic membrane; UMP, uridine-5'-phosphate; and ATP, adenosine triphosphate.

5 Standard uridine kinase conditions as described in Chart 1, except 5 mmole of UMP-2-14C (2 X 10^6 cpmp) replace uridine as substrate.

6 The assay conditions were as follows: 0.5 ml contained 5 mmole of UMP-2-14C (2 X 10^6 cpmp); 50 μmole of Tris buffer (pH 7.4); and enzyme (1.4 mg) (B differs from A by omission of ATP and Mg++)

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enhanced uridine kinase activity has been found in other tumors of the resultant tumor tissue should not be overlooked, since uridine kinase may be associated with the altered metabolism of the resultant tumor tissue should not be overlooked, since enhanced uridine kinase activity has been found in other tumors (10, 25).

We are currently trying to determine whether this enhancement of uridine kinase in RSV-infected CAM is of a qualitative or a quantitative nature.

Acknowledgments

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References


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