Inhibition of the Phosphorylation of Non-Histone Chromosomal Proteins of Rat Liver by Cordycepin and Cordycepin Triphosphate

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ABSTRACT

The effects of cordycepin (3'-deoxyadenosine) and cordycepin triphosphate on phosphorylation of non-histone chromosomal proteins were assessed in isolated hepatic nuclei in vitro. Cordycepin and cordycepin triphosphate competitively inhibited phosphorylation of urea-soluble nuclear proteins with a $K_i$ of $1.2 \times 10^{-3}$ and $8 \times 10^{-4}$, respectively. Isoelectric focusing of urea-soluble proteins indicated that inhibition occurred predominantly in nuclear proteins with isoelectric points of pH 4 to 7. Quaternary aminooethyl-Sephadex chromatography of extracts of nuclei incubated with cordycepin and cordycepin triphosphate also showed inhibition of phosphorylation of non-histone chromosomal proteins with similar isoelectric points, although greater resolution of proteins with isoelectric points of pH 6 to 7 was achieved. RNA polymerase I and II were not affected by cordycepin and cordycepin triphosphate after quaternary aminooethyl-Sephadex chromatography of nuclear extracts incubated with either agent. However, RNA polymerase I and II in isolated nuclei were competitively inhibited by cordycepin triphosphate but not by cordycepin. These results suggest that cordycepin triphosphate, and perhaps cordycepin too, may affect transcription via interference with the phosphorylation of non-histone chromosomal proteins.

INTRODUCTION

Cordycepin (3'-deoxyadenosine) is an effective inhibitor of polyadenylic acid synthesis in the nucleus and thus interferes with the processing of mRNA (5, 7, 20). Its inhibitor activity, however, also extends to the synthesis of other species of nRNA (6, 7, 15, 22, 24). In addition, cordycepin blocks the production of RNA viruses (27) and other species of nRNA (6, 7, 15, 22, 24). In addition, cordycepin is an effective competitive inhibitor of cyclic nucleotide-dependent protein kinases associated with the nucleus of normal and neoplastic liver (9, 13). These studies are the first to propose a metabolic target that is effectively inhibited by cordycepin per se and is not dependent on the formation of nucleotide metabolites, such as 3'-dATP (cordycepin triphosphate). Inhibition of the phosphorylation of NHCP (17, 23, 26), and the fact that liver hyperplasia (1, 2) and neoplastic transformation (3, 4, 16) are accompanied by increased phosphorylation of NHCP also suggests the importance of this process in regulating cell proliferation. For extension of the previous observations of the inhibition of nuclear protein kinases by cordycepin (9, 13), the effect of cordycepin on the phosphorylation of endogenous NHCP of isolated nuclei of rat liver was examined and forms the basis for this report.

MATERIALS AND METHODS

Materials. Cordycepin, Tris, ATP, UTP, CTP, GTP, and calf thymus DNA (type V) were obtained from Sigma Chemical Co., St. Louis, Mo., and 3'-dATP was purchased from Miles Laboratories, Elkhart, Ind. [y-32P]ATP (20.9 Ci/mmol) and [5,6-3H]UTP (39 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Ampholine ampholytes (pH 3.5 to 10) were purchased from LKB Instruments, Rockville, Md., and a-amanitin was obtained from Boehringer Mannheim Co., Indianapolis, Ind.

Animals. Male Sprague-Dawley rats (Taconic Farms, Germantown, N. Y.) weighing 200 to 500 g were maintained in cages with aspen shavings as bedding and allowed access to food and water as needed.

Isolation of Nuclei. Nuclei were prepared from rat liver by centrifugation in 2.2 M sucrose:10 mM MgCl₂ followed by washing on a 0.34 M sucrose:5 mM MgCl₂ gradient (11).

Phosphorylation of Proteins in Nuclei. Phosphorylation of proteins in isolated nuclei was carried out at 37°C for 5 min in a mixture (0.2 ml) containing 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM magnesium acetate, 5 μCi [y-32P]ATP (25 mCi/mmol), and 50 μl of nuclei (approximately 0.1 mg of urea-soluble protein). The reaction was terminated by the addition of 0.5 ml of 8 M urea:50 mM magnesium phosphate buffer (pH 7.6). The assay tubes were vortexed thoroughly and centrifuged at 10,000 x g in an Eppendorf centrifuge. The supernatant fluid was aspirated, and the extraction was repeated. Trichloroacetic acid-insoluble radioactivity was assayed on glass fiber filter discs as previously described (8). One unit of activity is defined as that amount of enzyme transferring 1 pmol of 32P to trichloroacetic acid-precipitable urea-soluble protein in 1 min at 37°C.

Phosphorylation of 200,000 x g Nuclear Extracts. Nuclei were disrupted with (NH₄)₂SO₄ and sonication and dialyzed overnight at 4°C against 1 liter of TGMED (9). Dialysates were

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centrifuged at 200,000 × g at 4° for 30 min, and the supernatant fluid was aspirated and used for further analyses.

Phosphorylation reactions were carried out at 37° for 10 min in a mixture containing 5 μCi of [γ-32P]ATP (0.5 Ci/mmol), 5 ml of 200,000 × g nuclear extract in TGMED, and either cordycepin or 3'-dATP as indicated. Following incubation, extracts were dialyzed at 4° against 1 liter of TGMED.

**QAE-Sephadex Chromatography.** Dialyzed 200,000 × g nuclear extracts were adsorbed to a column (0.9 × 15) of QAE-Sephadex A-25 equilibrated with TGMED. The column was washed with 10 ml of TGMED, and NHCP were eluted with a linear gradient of 0.1 to 0.5 M (NH₄)₂SO₄ in TGMED (8).

**Isoelectric Focusing.** Urea-soluble 32P-labeled proteins of isolated nuclei were dialyzed for 3 hr against 1 liter of 8 M urea and precipitated at -20° with 2% (w/v) potassium acetate in 95% ethanol and redissolved in 8 M urea.

QAE-Sephadex fractions of 32P-labeled 200,000 × g nuclear extracts were concentrated by vacuum dialysis against TGMED before dialysis against 1 liter of 8 M urea for 3 hr. Isoelectric focusing was carried out in polyacrylamide gels with ampholytes of pH 3.5 to 10 according to the method of Gronow and Griffiths (10), as modified by Lea et al. (18). Gels contained 100 to 200 μg of proteins in 8 M urea and were focused at 150 V constant voltage for 15 hr. The pH gradient was measured in a duplicate gel run in parallel for each experiment by soaking 5-mm slices in 2 ml of water. Gels were stained with Coomassie blue (10) and scanned at 650 nm in a Gilford spectrophotometer equipped with a linear transport.

**RNA Polymerase Assays.** RNA polymerase I and II were assayed in QAE-Sephadex fractions in a mixture (0.25 ml) containing: 50 mM Tris-HCl (pH 7.9); 1.6 mM MnCl₂; 1.6 mM dithiothreitol; 20 μg denatured DNA, 30 mM (NH₄)₂SO₄ (RNA polymerase I) or 70 mM (NH₄)₂SO₄ (RNA polymerase II); 0.6 mM each CTP, ATP, and GTP; and 0.01 mM [3H]UTP (0.4 Ci/mmol).

Assays with either 200,000 × g nuclear extract or nuclei were similar, except that reaction mixtures contained 50 mM (NH₄)₂SO₄ and were carried out in the presence (to estimate RNA polymerase I) or absence (RNA polymerase I and II) of 1 μg α-amanitin.

The Km for ATP was determined from double-reciprocal plots of the activity versus ATP concentration, and the Ki was calculated from the Michaelis-Menten equation for competitive inhibition.

**Protein Determination.** Protein concentrations were determined according to the method of Lowry et al. (19) after precipitation of samples with 10% trichloroacetic acid.

### RESULTS

**Inhibition by Cordycepin and 3'-dATP of the Phosphorylation of Urea-soluble Nuclear Proteins**

The inhibitory effect of cordycepin and 3'-dATP on the phosphorylation of nuclear proteins is shown in Chart 1. Inhibition was competitive with respect to ATP, and the Km for ATP was equal to 2.2 × 10⁻⁴ in the absence of inhibitor and was equal to 6.7 × 10⁻⁴ and 4.0 × 10⁻⁴ M in the presence of 2 × 10⁻³ M cordycepin and 5 × 10⁻⁵ M 3'-dATP, respectively. The calculated Ki's for cordycepin and 3'-dATP were 1.2 × 10⁻³ and 8 × 10⁻⁵ M, respectively.

**Isoelectric Focusing of Urea-soluble Nuclear Proteins.** Urea-soluble proteins were focused on 5% polyacrylamide gels over the pH range of 3.5 to 10.0 (Chart 2). Cordycepin and 3'-dATP inhibited the phosphorylation of all nuclear proteins, but particularly those with isoelectric points of pH 4 to 7.

**Phosphorylation of Nuclear Proteins Chromatographed on QAE-Sephadex**

To determine whether the phosphorylation of discrete NHCP was affected by cordycepin and 3'-dATP, we incubated 200,000 × g nuclear extracts with drug and subjected them to QAE-Sephadex chromatography (Chart 3). Two peaks of 32P-labeled phosphoproteins were resolved; one was eluted in the flowthrough fraction, and the other was eluted at 0.15 M (NH₄)₂SO₄ and coincided with RNA polymerase I (Fractions 11 to 13). No significant 32P radioactivity...
was found to be associated with RNA polymerase II (Fractions 29 to 31). Cordycepin (1 x 10^{-3} M) and 3'-dATP (1 x 10^{-4} M) inhibited 32P incorporation into the first phosphoprotein peak by 50% and into the second phosphoprotein peak by 30 to 50%.

The activities of RNA polymerase I and II were next assessed in nuclei or in nuclear extracts chromatographed on QAE-Sephadex. Nuclear extracts were incubated with either cordycepin or 3'-dATP and subsequently dialyzed and chromatographed on QAE-Sephadex. Neither RNA polymerase I nor II were inhibited by either drug following chromatography (Table 1), despite the observed inhibition of phosphorylation of NHCP under similar experimental conditions (Chart 3). In contrast, direct assays of RNA polymerase I and II in isolated nuclei in the presence of either cordycepin or 3'-dATP indicated that only the nucleotide was effective in inhibiting in vitro activity (Table 2). The inhibitory effect of 3'-dATP was competitive (Table 3) and exhibited a K_i of a magnitude similar to that found for the phosphorylation of NHCP (Chart 1).

Isoelectric focusing of the phosphoprotein peak coinciding with RNA polymerase I after QAE-Sephadex chromatography of extracts of nuclei that had been previously incubated with cordycepin or 3'-dATP is shown in Chart 4. A labeling pattern similar to that found for urea-soluble nuclear proteins (Chart 2) was found for the chromatographed NHCP, with the predominant inhibition by the adenosine analogs occurring in proteins with isoelectric points of pH 3.5 to 7. NHCP contained in the peak tube (Fraction 12)

Table 1
RNA polymerase activities after incubation of nuclear extract with cordycepin or 3'-dATP

RNA polymerase activities were assayed as described in "Materials and Methods." Nuclear extract from 33 g of liver was divided into 3 portions and incubated as described in Chart 3. After incubation, each extract was dialyzed against TGMED and was separately chromatographed on QAE-Sephadex. Activity represents the total incorporation in each extract (equivalent to 11 g of liver).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nuclear extract</th>
<th>QAE-Sephadex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I %</td>
<td>II %</td>
</tr>
<tr>
<td>None</td>
<td>791</td>
<td>100</td>
</tr>
<tr>
<td>Cordycepin (1 x 10^{-3} M)</td>
<td>856</td>
<td>108</td>
</tr>
<tr>
<td>3'-dATP (1 x 10^{-4} M)</td>
<td>853</td>
<td>109</td>
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Table 2
RNA polymerase activities in isolated nuclei after incubation with cordycepin or 3'-dATP

RNA polymerase activities were assayed in nuclei as described in "Materials and Methods," except that the concentration of ATP was varied, and 20 µg of denatured DNA was included where indicated.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ATP (M)</th>
<th>I %</th>
<th>II %</th>
<th>I %</th>
<th>II %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5 x 10^{-4}</td>
<td>33</td>
<td>100</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6 x 10^{-4}</td>
<td>48</td>
<td>100</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>Cordycepin (1 x 10^{-3} M)</td>
<td>5 x 10^{-4}</td>
<td>31</td>
<td>94</td>
<td>29</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>6 x 10^{-4}</td>
<td>47</td>
<td>98</td>
<td>30</td>
<td>83</td>
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<tr>
<td>3'-dATP (1 x 10^{-4} M)</td>
<td>5 x 10^{-4}</td>
<td>10</td>
<td>30</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>6 x 10^{-4}</td>
<td>35</td>
<td>73</td>
<td>35</td>
<td>97</td>
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</table>
Table 3
Effect of 3’-dATP on RNA polymerase I and II in isolated nuclei
RNA polymerase activities were assayed as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>RNA polymerase</th>
<th>Endogenous</th>
<th>Plus denatured DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km(ATP) (M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 10^{-4} M</td>
<td>2.4</td>
<td>5.3</td>
</tr>
<tr>
<td>3’-dATP (x 10^{-4})</td>
<td>1.9</td>
<td>2.8</td>
</tr>
<tr>
<td>K_i (x 10^{-4})</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Km/K_i</td>
<td>1.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Plus denatured DNA

Chart 5. Densitometric scans of nuclear proteins after isoelectric focusing. A, urea-soluble nuclear proteins; B, QAE-Sephadex fractions coinciding with RNA polymerase I.

Chart 4. Isoelectric focusing of nuclear proteins following QAE-Sephadex chromatography. Fractions coinciding with the peak of RNA polymerase I activity (equivalent to Fractions 11 to 13 in Chart 3) were pooled and concentrated before isoelectric focusing as described under "Materials and Methods." O, •, and A, ^32P radioactivity.

DISCUSSION

Cordycepin previously has been shown to be an effective competitive inhibitor of cyclic nucleotide-independent and -dependent protein kinases (9,13). In this study, cordycepin and 3’-dATP competitively inhibited the phosphorylation in vitro of NHCP from rat liver. Isoelectric focusing showed that the major portion of this effect resided in proteins of isoelectric points of pH 4 to 7. Experiments designed to assess this effect after QAE-Sephadex chromatography of nuclear extracts revealed similar results. An interesting feature of the latter experiments was the coincidence of

incorporated ^32P into NHCP and the activity of RNA polymerase I, a result in agreement with those of Hirsch and Martelo (12). However, the low specific activity of RNA polymerase after this step of purification does not rule out the possibility that other acidic nuclear proteins in this fraction are phosphorylated. Nevertheless, no inhibitory
effect by cordycepin or 3′-dATP on RNA polymerase I and II was sustained after dialysis and QAE-Sephadex chromatography, which suggests that cordycepin does not directly interfere with polymerase activities and that both drugs are not tight-binding inhibitors. This was further corroborated by the lack of a direct effect of cordycepin on RNA polymerase activities in isolated nuclei; however, 3′-dATP did show significant inhibitory activity that was competitive with ATP. These results suggest that only 3′-dATP is a direct competitive inhibitor of RNA polymerases as well as of phosphorylation of NHCP, while cordycepin only directly affects the latter. On the basis of these results, it would appear that, if cordycepin per se has an inhibitory effect on RNA synthesis in vivo, it is an indirect one that may be related to its action on the phosphorylation on NHCP.

Although the precise relationship between the phosphorylation of NHCP and transcription is not yet definitive, it is known that the rate of RNA synthesis in eukaryotic cells with homologous DNA as template is increased with increasing amounts of phosphorylated NHCP (23). The in vitro inhibition of protein kinases by cordycepin (9, 13) and the in vitro inhibition of the phosphorylation of NHCP by cordycepin and 3′-dATP suggest that cordycepin could act in vivo either directly or through the nucleotide metabolite by inhibiting the phosphorylation of NHCP required for gene transcription. This mechanism may be coincident with or in addition to other processes such as inhibition of the synthesis of polyadenylic acid (5, 7). However, impairment in the maturation of precursor RNA (7, 24) may be due, at least in part, to interference with NHCP factors regulating transcription by RNA polymerase I. The concentrations of cordycepin and 3′-dATP required to inhibit phosphorylation of NHCP are within the range necessary to exert such an effect (5, 6, 15, 24).

REFERENCES

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