Phosphorylation of 3-Deazaguanosine by Nicotinamide Riboside Kinase in Chinese Hamster Ovary Cells

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ABSTRACT

The growth inhibitory activity of 3-deazaguanosine toward a mutant line (TG³-3) of Chinese hamster ovary cells deficient in hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) was substantially reversed by the simultaneous addition of nicotinamide riboside. The activities of most other ribonucleoside analogues tested were unaffected. The formation of cellular 3-deazaGMP and 3-deazaGTP from the ribonucleoside analogue, as measured by high-pressure liquid chromatography, was inhibited by the presence of nicotinamide riboside. The inhibition was dependent on concentration of 3-deazaguanosine and could also be demonstrated by following the metabolism of 3-deazaguanosine, labeled with ¹⁴C in the ribose moiety, to [¹⁴C]3-deazaGTP. In the presence of 100 μM nicotinamide riboside formation of the labeled triphosphate derivative of 3-deazaguanosine was undetectable.

A 3-deazaguanosine phosphorylating activity was separated from other cellular kinases by DEAE-cellulose chromatography. Contaminating purine nucleoside phosphorylase (EC 2.4.2.1) was subsequently removed by sucrose density gradient centrifugation. The resulting enzyme preparation demonstrated the greatest activities with nicotinamide riboside and 3-deazaguanosine and, in addition, could also phosphorylate tiazofurin and guanosine to lesser, but significant, degrees.

These and other observations suggest that 3-deazaguanosine, and perhaps other agents such as tiazofurin, may, at least in part, be phosphorylated by a nicotinamide ribonucleoside kinase in these cells. If so, it is possible that the activity of this agent in other types of cells in vitro could be dependent upon the presence of this enzyme and that it could be influenced by cellular concentrations of the natural pyridine nucleoside.

INTRODUCTION

3-Deazaguanosine (1) is unique among guanosine analogues in demonstrating inhibitory activity toward cell lines deficient in hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) which are, accordingly, highly resistant to the base, 3-deazaguanine (2). This activity has been attributed to the direct anabolism of the analogue nucleoside to phosphorylated derivatives (3), a phenomenon not previously observed with guanosine or other analogues of it; these compounds are generally degraded by purine nucleoside phosphorylase (EC 2.4.2.1) producing the base which is then available for salvage by hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8). Although 3-deazaguanosine is reportedly a substrate for purine nucleoside phosphorylase (EC 2.4.2.8), it is a relatively poor one (4), presumably making it more available than guanosine or other analogues for other cellular reactions.

Unpublished competition studies from this laboratory have suggested that 3-deazaguanosine and tiazofurin may be phosphorylated by the same enzyme. Since tiazofurin (as the mono-

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: CHO, Chinese hamster ovary; HPLC, high-pressure liquid chromatography; tiazofurin, 2-β-d-ribofuranosylthiazole-4-carboxamide; ribavirin, 1-β-d-ribofuranosyl-1.2.4-triazole-3-carboxamide; MIC, minimum inhibitory drug concentration; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; NNM, nicotinamide mononucleotide.
HPLC Fractionation of Metabolites. Four fractionation methods were used. In the first method, (method A), nicotinamide and related metabolites were separated using a Waters Associates (Milford, MA) high-pressure liquid chromatography equipped with two Model 510 pumps, System Controller, and a column of Partisil-10 SAX anion exchange resin (25 x 4.6 mm; Whatman Inc.). Extracts were injected with the U6k injection system and eluted with a concave gradient (curve 9 on the System Controller) of ammonium phosphate from 100% 0.005 M, pH 2.8, to 0.3 M KC1 in buffer A) and approximately 90 fractions containing the radioactive peak, appearing in the first 5 ml of eluate, were collected and lyophilized to reduce the volume. A second incubation was then carried out with a mixture containing the radiolabeled NMN, 35 mM Tris-HCl, pH 7.8, and 5 units of nucleotide pyrophosphatase in a final volume of 1.45 ml. The mixture was incubated for 45 min at 37°C and then the reaction was terminated by the addition of perchloric acid to 0.4 N. After removal of the precipitate by centrifugation and neutralization with KOH to pH 8, the mixture was applied to a 2-ml column of AG 1x8-formate (Bio-Rad Laboratories) and the radioactive NMN eluted with H2O. Fractions containing the radioactive peak, appearing in the first 5 ml of eluate, were combined and lyophilized. Occasionally the product was lyophilized and analyzed again for purity by HPLC, however, substantial degradation occurred during this procedure.

RESULTS

The HGPRT-deficient cell line, TG8-3, is highly resistant to 3-deazaguanine but demonstrates only partial resistance to the nucleoside, 3-deazaguanosine (3). This suggests that, in wild-type CHO cells, there are two pathways of activation of the nucleoside, one via purine nucleoside phosphorylase with subsequent phosphoribosylation of the base by HGPRT and a second by another mechanism that is responsible for the toxicity observed toward HGPRT-deficient cells.

The growth-inhibitory activity of antimetabolites to CHO cells can conveniently be estimated by allowing cells to form clones in the presence of varying concentrations of drug. In this type of assay, the growth inhibitory activity of 3-deazaguanosine to HGPRT-deficient cells (line TG8-3) is highly reproducible and appears to be substantially influenced only by the presence of externally added nicotinamide riboside (Table 1). Aminoimidazole carboxamide riboside had a very slight, but consistent, effect while all other natural nucleosides, as well as nicotinamide and guanine, had no apparent influence on the minimum inhibitory concentration of the agent. This type of experiment is convenient for estimating the activities of numerous compounds, however to obtain a more quantitative evaluation of the reversal effect of nicotinamide riboside the experiment shown in Fig. 1 was carried out. In this experiment growth in 25-ml flasks was allowed for 7 days after which the cells were detached with trypsin and counted directly. The reversal, by nicotinamide riboside, of 3-deazaguanosine activity toward TG8-3 cells appeared slightly less dramatic when determined in this way, presumably because of utilization or degradation of the nicotinamide riboside by the larger number of cells (higher ratio of cells to volume of medium). Degradation of

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nicotinamide riboside at concentrations up to 100 \(\mu M\) in CHO cells. A slight, but detectable, reversal effect was seen with tiazofurin, an agent whose metabolism and activity may be dependent upon at least one enzyme of the NAD biosynthetic pathway. NAD pyrophosphorylase (EC 2.7.7.1) has been shown to be able to catalyze the formation of the active metabolite of tiazofurin, tiazofurin adenine dinucleotide, from tiazofurin 5'-monophosphate, and ATP (5). Although 3-deazaguanine is utilized primarily via HGPRT in CHO cells, the partial reversal of 3-deazaguanine inhibitory activity by 100 \(\mu M\) nicotinamide riboside in these cells could reflect a small amount of metabolism of this compound via the nucleoside, the route which appears sensitive to nicotinamide riboside. On the other hand, since the partial reversal occurs only at high nicotinamide riboside concentration, it could result from production of sufficient nicotinamide to compete with 3-deazaguanine for cellular 5-phosphorylribose-1-pyrophosphate. Reversal of 3-deazaguanosine activity is most pronounced in TG\(^*\)-3 cells which are unable to utilize 3-deazaguanine and are consequently highly resistant to it as well as to 6-thioguanine and 6-thioguanosine.

It is possible that 3-deazaguanosine could be interfering somehow with cellular NAD synthesis, producing a requirement that could be satisfied by nicotinamide ribonucleoside or NAD. To test this possibility, the effect of 3-deazaguanosine on \(^{14}C\)nicotinamide metabolism to NAD was determined (not shown). 3-Deazaguanosine at 100 \(\mu M\) had essentially no effect on incorporation of label from \(^{14}C\)nicotinamide into NAD, the latter presumably reflects dilution of label resulting either from phosphorylation of the nucleoside to form nicotinamide or at the level of NMN, formed by direct phosphorylation of the nucleoside. NAD levels, judged by absorbance, were unaffected by 3-deazaguanosine but appeared considerably higher in the presence of nicotinamide ribonucleoside, suggesting that the latter may be efficiently metabolized to NAD in these cells.

The formation of the triphosphate derivative from 3-deazaguanosine in cultured cells, as reported earlier (3), provides a tool to assess the effects of nicotinamide riboside on this conversion. The hypoxanthine-guanine phosphoribosyltransferase-deficient mutant, TG\(^*\)-3, was exposed to 100 \(\mu M\) 3-deazaguanosine and varying concentrations of nicotinamide riboside followed by HPLC analysis of the nucleotide pools (Fig. 2). The amount of 3-deazaGTP formed appeared inversely proportional to the concentration of exogenously supplied nicotinamide riboside. A measurable effect was seen at concentrations of nicotinamide riboside as low as 5 \(\mu M\) (Fig. 2, graph 3) and the maximal effect at approximately 50 \(\mu M\) (Fig. 2,
Inhibition of 3-deazaGTP formation from 3-deazaguanosine by nicotinamide riboside in cultured TG*-3 cells. Monolayer cultures of TG*-3 cells were incubated, as described for metabolic experiments in "Materials and Methods" with 100 µm 3-deazaguanosine and the following concentrations of nicotinamide riboside: graph 2, none; graph 3, 5 µM; graph 4, 10 µM; graph 5, 50 µM; and graph 6, 100 µM. Graph 1 nucleotide profile from cells incubated without drug. Nucleotides were extracted as described in "Materials and Methods" and 2 x 10⁶ cell equivalents were analyzed by HPLC method C.

These observations suggest that nicotinamide riboside (or NMN) competes at some step in the cellular utilization of 3-deazaguanosine rather than satisfying a requirement created by it. This was further demonstrated by the experiment of Fig. 3 in which metabolism, to the triphosphate derivative, of 3-deazaguanosine (0.52 µM) having a ¹⁴C label in the ribose moiety was completely inhibited by the presence of 100 µM nicotinamide riboside. The smaller nucleotide peak at 31 min that is unaffected by nicotinamide riboside may reflect reutilization of [¹⁴C]ribose-1-phosphate formed by the limited action of purine nucleoside phosphorylase on [¹⁴C]-3-deazaguanosine. The unique ability of nicotinamide riboside to affect metabolism of 3-deazaguanosine is illustrated in Table 3. Neither NAD itself, nor the precursors nicotinamide and nicotinic acid demonstrated inhibitory activity.

If it is assumed that 3-deazaGTP arising from metabolism of 3-deazaguanine is formed by the same route as that from the nucleoside (other than the initial step), one can conclude from these experiments that nicotinamide riboside appears either to compete with 3-deazaguanosine for phosphorylation or to inhibit the reaction. If this is indeed the case it provides a tool to determine if a phosphorylating activity observed in cell extracts is in fact relevant to the action of the drug in whole cells, i.e., nicotinamide riboside should interfere with phosphorylation. The existence of such an activity was demonstrated in crude extracts of TG*-3 cells (Table 4); formation of [¹⁴C]-3-deazaGMP from [ribofuranosyl-¹⁴C]-3-deazaguanosine was dependent upon ATP and was inhibited by the presence of nicotinamide riboside.

To determine if the 3-deazaguanosine-phosphorylating activity was related to other cellular purine salvage enzymes, a crude CHO extract was fractionated by DEAE-cellulose chromatography (Fig. 4). This procedure, which is described elsewhere (8), effectively separates adenosine kinase, hypoxanthine-guanine phosphoribosyltransferase, purine nucleoside phosphorylase and deoxycytidine kinase (Fig. 4, top). The apparent 3-deazaguanosine-phosphorylating activity eluted in the region of purine nucleoside phosphorylase. In the experiment shown here the fractions in the earlier and later parts of the profile were not analyzed in order to conserve a limited supply of

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**Table 3** Effect of NAD and its precursors on metabolism of 3-deazaguanosine (100 µM) to 3-deazaGTP in TG*-3 cells

<table>
<thead>
<tr>
<th>Additions (100 µM)</th>
<th>3-DeazaGTP formed (pmol)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>300 ± 60</td>
</tr>
<tr>
<td>Nicotinamide riboside</td>
<td>&lt;50</td>
</tr>
<tr>
<td>NAD</td>
<td>295 ± 5</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>310 ± 40</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>450 ± 10</td>
</tr>
</tbody>
</table>
3-DEAZAGUANOSINE METABOLISM

Table 4 Effect of nicotinamide riboside on [14C]-3-deazaguanosine phosphorylation by a crude extract of TGK-3 cells

A crude extract of logarithmically growing TGK-3 cells was prepared as described in “Materials and Methods” and used directly without dialysis. Reaction mixtures were as described for assaying [14C]-3-deazaguanosine phosphorylation. Reaction was terminated after 2 h incubation at 37°C by the addition of 1 ml 0.4 N perchloric acid. After 10 min in ice the precipitates were removed by centrifugation and the supernatants neutralized with KOH. Aliquots (0.5 ml) were analyzed by HPLC method C for 3-deazaGTP formation and by method D for 3-deazaGMP formation. These data are representative of several experiments that were carried out.

<table>
<thead>
<tr>
<th>Composition of reaction mixture</th>
<th>3-DeazaGMP formed</th>
<th>3-DeazaGTP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>2,640</td>
<td>5.79</td>
</tr>
<tr>
<td>Without ATP</td>
<td>467</td>
<td>1.02</td>
</tr>
<tr>
<td>With 100 μM nicotinamide riboside</td>
<td>335</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Fig. 4. DEAE-cellulose chromatography of known purine salvage enzymes (top) and apparent 3-deazaguanosine phosphorylating activities (bottom) in CHO cell extracts. Salvage enzymes were analyzed by methods previously described (1, 7). Extract from 3 ml of packed CHO cells was applied to the column for the upper graph and 2.2 ml cells was used for the lower graph. 3-Deazaguanosine phosphorylation was measured using DE81 disks as described in “Materials and Methods” using 1 μM [ribosyl-14C]-3-deazaguanosine as substrate. Aliquots (25 μl) of the indicated column fractions were analyzed. Bottom, △, apparent 3-deazaguanosine phosphorylation; □, phosphorylation of deoxyxycytidine.

[14C]-3-deazaguanosine. Experiments prior to this one, however, showed no other peaks of 3-deazaguanosine phosphorylating activity.

The active fractions from DEAE-cellulose chromatography were pooled and the phosphorylating activity was further purified by sedimentation through a sucrose density gradient to separate it from purine nucleoside phosphorylase, a relatively large protein (Fig. 5). The apparent phosphorylating activity (i.e., activity resulting in formation of a labeled product adsorbing to DE81 disks) was resolved into two peaks, labeled A and B in Fig. 5 (graph 1). The activity observed in peak A was inhibited by the addition to the incubation mixtures of a high concentration of nicotinamide riboside (Fig. 5, graph 2); peak B was unaffected. Conversely, the addition of inosine to the incubation mixtures eliminated peak B but did not affect peak A (Fig. 4, graph 3). The peaks of activity catalyzing phosphorylation of 3-deazaguanosine (Fig. 5, graph 1, peak A) and tiazofurin (Fig. 5, graph 4) remained together, apparently unaffected by the removal of purine nucleoside phosphorylase. The identities of the products formed from [ribosyl-14C]-3-deazaguanosine by the two peaks of activity were verified by HPLC (Table 5). Peak A produced [14C]-3-deazAGM while peak B, presumably purine nucleoside phosphorylase, produced [14C]ribosyl-1-phosphate, exclusively.

The characteristics of the reaction catalyzed by the peak A enzyme fraction, with [H]guanosine as substrate, are shown in Fig. 6. There is a clear requirement for ATP; IMP does not support the reaction (not shown). Addition of 100 μM nicotinamide riboside at the beginning of the reaction prevented formation of GMP; addition after 60 min incubation prevented additional formation of GMP but had no effect on that which had already been formed, indicating that the preparation is free of 5′-nucleotidase activity.
A variety of radiolabeled nucleosides were tested for substrate activity with the preparation (Table 6). Clearly the most efficient were nicotinamide riboside and 3-deazaguanosine (tested at much lower concentration than the others owing to the method of its preparation). Substantial activity was also observed with tiazofurin and guanosine. The products of the reactions with these compounds were identified, by HPLC, to be tiazofurin 5'-monophosphate and GMP. The scarcity of radiolabeled 3-deazaguanosine has precluded determination of kinetic constants.

Although it is clear that nicotinamide riboside does not have an obligatory role in NAD synthesis, Table 7 demonstrates that CHO cells are capable of utilizing it for the synthesis of NAD. A relatively short incubation (3 h) was carried out for this experiment to minimize the amount of nicotinamide riboside phosphorylyzed to nicotinamide; the addition of unlabeled nicotinamide did not dilute the amount of label incorporated into NAD. On the other hand, addition of 30 μM unlabeled nicotinamide riboside was added after 60-min incubation (arrow); δ, complete reaction mixture containing 50 μM nicotinamide riboside.

DISCUSSION

The unique activity of 3-deazaguanosine to HGPRT-deficient cells, compared to other guanosine analogues, appears to reflect its direct phosphorylation to the nucleotide level by a nicotinamide riboside phosphokinase. The observation that nicotinamide riboside prevented conversion of 3-deazaguanosine to nucleotide derivatives both in growing cells and in cell extracts in addition to preventing cytotoxicity suggests that the reversal phenomenon is not related to permeation. Additionally, it implies that the 3-deazaguanosine-phosphorylating activity demonstrated here is relevant to the cytotoxic (and presumably the antitumor) activity of 3-deazaguanosine. We have resolved an enzyme fraction in CHO cells that phosphorylates 3-deazaguanosine and a variety of other nucleosides; the best substrate, however, is nicotinamide riboside.

The general conclusion from these studies is that 3-deazaguanosine enters cell metabolism primarily via phosphorylation by a pyridine ribonucleoside phosphokinase such as that described by Nishizuka and Hayaishi (11). The latter enzyme was partially purified from bovine liver and was shown to react with the ribonucleoside of either nicotinate or nicotinamide. The importance of this enzyme in cellular NAD metabolism is not clear but it can probably be assumed to function in a salvage capacity. The ribonucleosides of nicotinate and nicotinamide have received minimal attention in the literature. They have, however, been shown to occur metabolically in several systems (12–15). A study of pyridine nucleotide synthesis by cultured cell lines, carried out by Hillyard et al. (15) suggested that nicotinamide riboside was an intermediate in the biosynthesis of NAD from nicotinamide. Jacobson et al. (16) have, however, concluded that nicotinamide riboside is not an intermediate in the conversion of nicotinate to NAD in cultured BALB 3T3 cells and suggest that in those studies demonstrating an involvement of the ribonucleoside there may have been confusion with 1-methylnicotinamide which has very similar chromatographic properties (17). Though, the ribonucleosides of nicotinate and nicotinic acid have been observed in a variety of cells and tissues, the role of these compounds in NAD metabolism and their relative importance remain very unclear and may differ from one cell type to another.

The interaction of a guanosine analogue with an enzyme associated with NAD metabolism has not previously been reported. While the full significance of this observation remains to be determined, it does indicate that the presence of nicotinamide riboside, and that of pyridine ribonucleoside kinase, may be important determinants of the potential antitumor activity.
of 3-deazaguanosine. Since tiazofurin was also phosphorylated by this enzyme fraction, it is possible that metabolism of nicotinamide riboside may be a determinant in the action of this and other agents as well.

Another interesting aspect of this study is the phosphorylation of guanosine exhibited by the enzyme fraction described here. Although inosine-guanosine kinase activities from mammalian cells have been reported (18-19), the existence of such an enzyme has remained controversial. The demonstration here that a nicotinamide ribonucleoside phosphokinase is capable, in the absence of purine nucleoside phosphorylase, of phosphorylating guanosine may provide an explanation for the earlier observations.

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