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Activation of deoxycytidine kinase by inhibition of DNA synthesis in human lymphocytes

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Abstract

Deoxycytidine kinase (dCK, EC.2.7.1.74) is a key enzyme in the intracellular metabolism of 2-chlorodeoxyadenosine, 1-β-D-arabinofuranosylcytosine, difluorodeoxycytidine, and other drugs used in chemotherapy of different leukaemias and solid tumours. Recently, stimulation of dCK activity was shown by these analogues and by other genotoxic agents such as etoposide and NaF, all of which cause severe inhibition of DNA synthesis in cell cultures. Here we describe that direct inhibition of DNA polymerases by aphidicolin stimulated dCK activity in normal lymphocytes and acute myeloid leukaemic cells, as well as in HL 60 promyelocytic cell cultures. Increased dCK activity was not due to new protein synthesis under our conditions, as measured by immunoblotting. Partial purification by diethylaminoethyl–Sephadex chromatography revealed that the activated form of dCK survived purification procedure. Moreover, it was possible to inactivate purified dCK preparations by recombinant protein phosphatase with Ser/Thr/Tyr dephosphorylating activity. These data suggest that the activation of dCK may be due to phosphorylation, and that deoxynucleoside salvage is promoted during inhibition of DNA synthesis in human lymphocytes.

Keywords: Human; Deoxycytidine kinase; Aphidicolin; Lymphocyte; Phosphorylation; DNA synthesis

1. Introduction

dCK (EC 2.7.1.74) is a cytoplasmic enzyme [1], although it was shown in the nucleus under overexpression [2,3] and is highly expressed in lymphoid tissues [4]. The enzyme phosphorylates purine and pyrimidine deoxynucleosides, using ATP or UTP as a phosphate donor in a process requiring a conformational change during catalysis [5]. Beside its natural substrates, dCK activates a large number of deoxynucleoside analogues, such as 1-β-D-arabinofuranosylcytosine (araC), 2′,2′-difluorodeoxycytidine (dFdC, gemcitabine), 2-fluoro-9-β-D-arabinosyladenine (FaraA), CdA (cladribine), and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA), used in chemotherapy. The phosphorylated deoxynucleoside analogues interact with several enzymes in the salvage pathway of DNA precursors, including deoxycytidylate deaminase [6,7] ribonucleotide reductase [8,9], and others like DNA methyltransferase [10], leading in most cases to incorporation of the analogue into the DNA and hence to the inhibition of DNA synthesis and cell growth. The level of dCK activity is one of the factors determining the sensitivity of different leukaemias and solid tumours to deoxynucleoside analogue toxicity [11–16]. Therefore, investigations into the regulation of dCK have a high impact on leukaemia and cancer research.

Previous studies have demonstrated that dCK can be activated by short-term incubation of human lymphocytes with CdA [17]. Beside CdA, other deoxynucleoside analogues, as well as etoposide, also promoted dCK activity in lymphocytes and in HL 60 cell cultures [18]. Recently, it was also shown that the disturbance of nucleotide pools by

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Abbreviations: dCK, deoxycytidine kinase; dTK, thymidine kinase; CdA, 2-chloro-2′-deoxyadenosine; araC, 1-β-D-arabinofuranosylcytosine; ^{3}HdC, 2′-deoxy-[5-^{3}H] cytidine; ^{3}HdT, 2′-deoxy-[5-methyl-^{3}H] thymidine; HL 60, human promyelocytic cell line; AML, acute myeloid leukaemia; PBMC, peripherial blood mononuclear cell; APC, aphidicolin; and DEAE, diethylaminoethyl.

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exposure of cells to NaF also stimulated dCK [19]. All these compounds are genotoxic, probably causing a perturbance in nucleotide metabolism and an inhibition of DNA synthesis. The question addressed in this paper is whether direct inhibition of DNA polymerases by APC results in similar activation of dCK.

2. Materials and methods

2.1. Chemicals

^{3}HdT (37 MBq/mL), ^{3}HdC (37 MBq/mL), and the Western blotting detection system were from Amersham Life Sciences. Recombinant λ-protein phosphatase was the product of Calbiochem. CdA was synthesised by Z. Kazimierczuk [20]. All other chemicals were purchased from Sigma.

2.2. Cells and treatment

The HL 60 promyelocytic cell line and primary cultures of PBMC from normal individuals, from patients with AML [21], and from tonsils of 3- to 6-year-old children [22] were prepared as described earlier. Cells were treated with indicated concentrations of APC, CdA, and NaF in serum-free Eagle's minimal essential medium for 2 hr.

2.3. Assay of DNA synthesis

Cell cultures treated with the indicated amounts of APC were washed with warm medium and pulse-labelled either with ^{3}HdT or with ^{3}HdC (1.0 μCi/mL) for 20 min. Radioactive precursors incorporated into the DNA were quantified in the 0.5 N perchloric acid insoluble fraction of the cells [22].

2.4. Assay of dCK and dTK enzyme activity, partial purification, and immunostaining of dCK

Crude extracts of the lymphocyte cultures were prepared in 5 mL 50 mM Tris–HCl buffer, pH 7.6 containing 2 mM dithiothreitol, 21% glycerol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulphonyl fluoride by homogenisation and by freezing-thawing in liquid N_2 three times. Partial purification of dCK included a centrifugation step at 104,000 *g* at 4° for 1 hr and a DEAE–Sephadex column chromatography. About 20 mg protein in 4 mL volume was applied to the DEAE–Sephadex A-25 column, equilibrated with buffer A (50 mM imidazole buffer [pH 7.0] containing 5 mM $MgCl_2$, 4 mM β-mercaptoethanol, 20% ethyleneglycol). Elution was performed with a linear 0–1 M KCl gradient in the same buffer. For the determination of dCK activity, fractions were reactivated by a 20-min incubation with 50 mM dithiothreitol and 1 mg/mL of BSA before the enzyme activity measurements [23]. dCK and dTK activities were determined both in the crude extracts and in the DEAE–Sephadex fractions under optimal assay conditions and substrate saturation [24]. Electrophoretic separation, Western blotting, and immunostaining were performed as described earlier [17] using either affinity-purified primary rabbit antibody against human dCK [25] or by rabbit antibody raised against the C-terminal peptide of human dCK [2]. In both cases, purified recombinant dCK was applied as standard [4].

2.5. Protein phosphatase treatment of dCK preparations

Recombinant dCK and peak fractions from the ion-exchange column were treated with λ recombinant protein phosphatase (4 unit/μL) in a 50 mM Tris–HCl buffer (pH 7.8), 1 mg/mL of BSA, 10 mM dithiothreitol, and 2 mM $MnCl_2$ in the presence or absence of 1 mM Na_3VO_4 (an inhibitor of protein phosphatase), at 30°, for different time periods as indicated. Then the reaction mixture was diluted up to a low Mn^{2+} concentration to inactivate protein phosphatase.

3. Results

3.1. Stimulation of dCK activity by aphidicolin treatment of lymphocytes

The results presented in Fig. 1A demonstrate that less than 5 μM APC inhibited ^{3}HdT incorporation by more than 90% in primary cultures of tonsillar lymphocytes. The rate of ^{3}HdC incorporation also decreased (Fig. 1A), but not as much as ^{3}HdT incorporation. One possible reason why ^{3}HdC incorporation decreases less than ^{3}HdT incorporation could be a higher rate of labelled deoxycytidine supply in the presence of APC. dCK activity was clearly elevated after APC treatment (Fig. 1B). The maximum level of dCK activity was 2- to 3-fold higher than the control level. Inhibition of DNA synthesis by APC, however, did not change dTK activity (Fig. 1B). dCK activity, measured under optimal conditions and at substrate saturation in the crude cell extracts, was about 4–10 pmol/(min × 10^6 cells), while less than 1 pmol/(min × 10^6 cells) ^{3}HdC was phosphorylated in the cell cultures, suggesting a limited substrate supply in cell cultures.

The stimulatory effect of APC on dCK activity was also investigated in normal (Fig. 2A, PBMC) and leukaemic peripheral lymphocytes (Fig. 2B, AML) as well as in promyelocytic HL 60 cells. These cells had different activities of dCK:PBMC: 2–3 pmol/(min × 10^6 cells), AML: 4–8 pmol/(min × 10^6 cells), HL 60: 20–30 pmol/(min × 10^6 cells), and all had elevated dCK activities after incubation with APC. However, the extent of dCK activation was slightly different, as a higher dCK level was associated with a smaller stimulation of dCK activity.

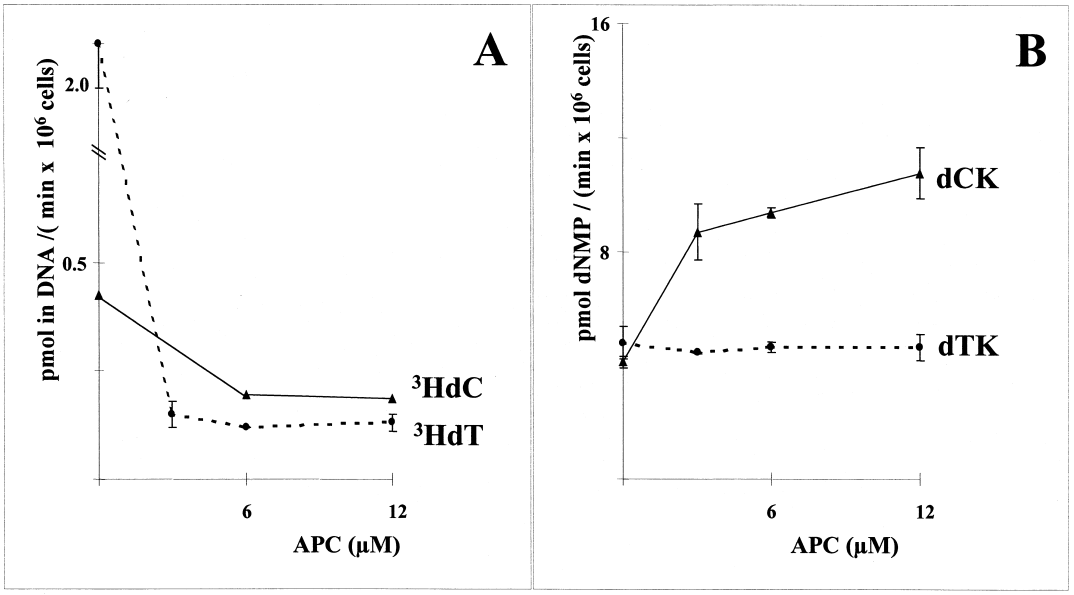

Fig. 1. Activation of deoxycytidine kinase during inhibition of DNA synthesis by aphidicolin. (A) Parallel cultures of tonsillar lymphocytes were incubated in the presence of indicated concentrations of APC for 2 hr. then cells were pulse-labelled either with ^{3}HdT, (●----●) or with ^{3}HdC (▲——▲) for 20 min. Incorporation of radioactive nucleosides into the acid-precipitable fraction of cells was measured as described in Methods. (B) dCK (▲——▲) and dTK (●----●) activities were measured in crude extracts of cell cultures treated with APC as described under (A). The figure illustrates mean values of three independent experiments; standard errors were <20%.

3.2. Purification of dCK from stimulated and control cells

Partial purification of the enzyme was performed to gain more insight into the molecular mechanism of dCK activation. Crude cell extracts of control and APC-treated cells were purified by ultracentrifugation and DEAE–Sephadex chromatography with KCl gradient elution as described earlier [26]. Fig. 3 shows a typical profile of dCK activity as measured by DEAE–Sephadex chromatography and KCl gradient elution from control and APC-treated lymphocytes. The elution profiles of activated and control extracts did not differ significantly. dCK activity was measured in the presence of 1 mM thymidine to inhibit thymidine kinase 2 activity [27]. Partial purification of dCK was also performed

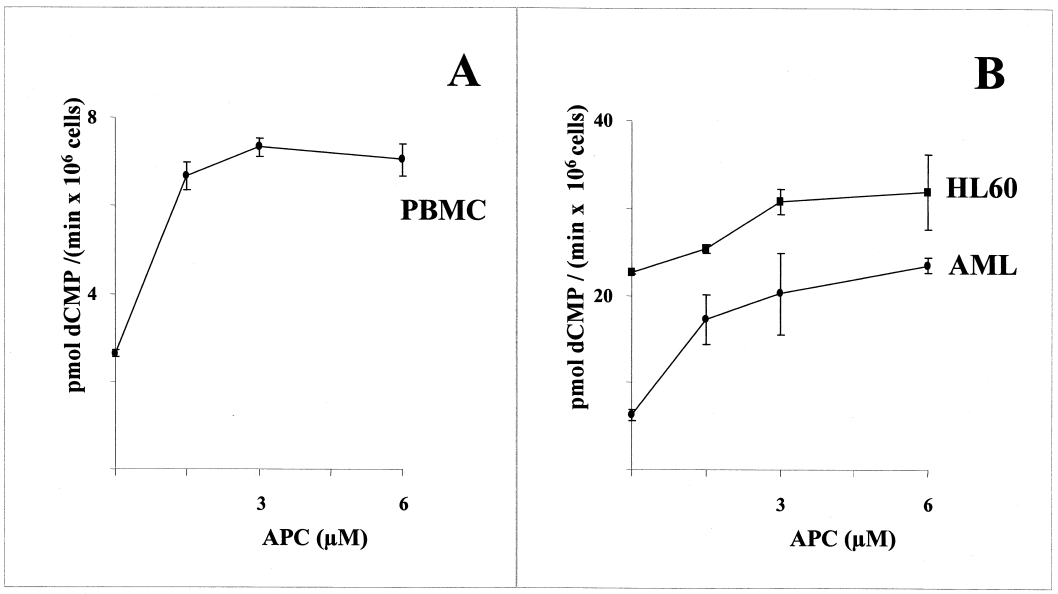

Fig. 2. Activation of deoxycytidine kinase in different cells by APC. Peripheral blood mononuclear cells from normal individuals (A) and from patients with acute myeloid leukaemia, as well as the HL 60 cell line (B), were incubated with varying concentrations of APC for 2 hr. After incubation, cells were washed twice, and crude cell extracts were prepared from the cell pellets and used for the assay of dCK activity. The figure illustrates mean values of three independent experiments; standard errors were <25%.

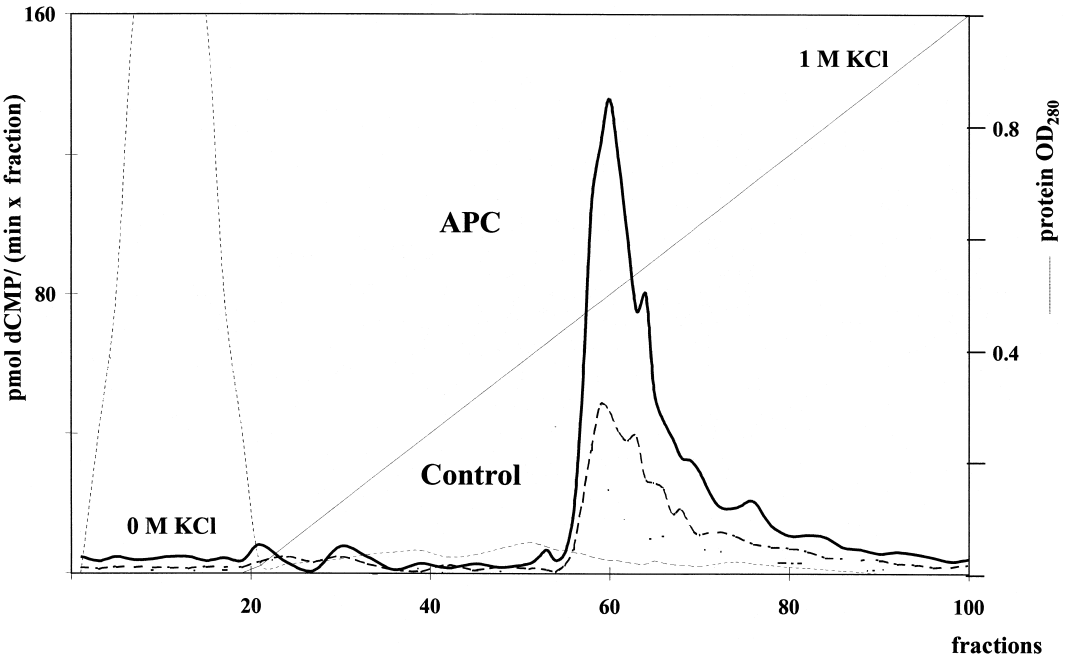

Fig. 3. DEAE–Sephadex chromatography from control and aphidicolin-treated cells. Lymphocytes were incubated with or without 6 μM APC for 2 hr. Crude cell extracts were ultracentrifuged and loaded onto the DEAE–Sephadex column. dCK was eluted with a KCl gradient, and the fractions were measured for dCK activity in the presence of 1 mM thymidine (continuous line: dCK activity in APC-treated cells; broken line: dCK activity in control cells, light, dotted line: $O.D._{280}$; light, continuous line: KCl gradient).

from extracts of lymphocytes incubated in the presence of 1 μM CdA, 6 μM APC, or 15 mM NaF for 2 hr. dCK activity was measured in the crude cell extracts and in the peak fractions of DEAE–cellulose chromatography (Table 1). dCK protein levels were also determined in the same samples by immunoblotting (Fig. 4). The dCK activity was enhanced in all the purified preparations to a similar extent as in the crude extracts, but there was no significant difference in the dCK protein level either among the crude extracts (Fig. 4, lanes 5a–8a) or among the purified preparations (Fig. 4, lanes 5b–8b). Recombinant dCK was used as standard for the Western blotting procedure (Fig. 4, lanes 1–3), with a somewhat higher molecular weight because of the His-tag fused to dCK.

Table 1
Deoxycytidine kinase activities of crude and purified extracts from tonsillar lymphocytes

Treatments	Crude extracts		Extracts purified by DEAE–Sephadex	
	pmol dCMP/(mg protein × min)			
	AVG	STD	AVG	STD
Control	**234**	22	**3131**	290
CdA	**435**	39	**5386**	485
APC	**570**	48	**9280**	870
NaF	**382**	41	**5248**	512

Specific enzyme activities are shown (AVG: average of 3 determinations, STD: standard deviation).

3.3. Inactivation of purified dCK by protein phosphatase treatment

The experiment shown in Fig. 5A revealed that purified dCK preparations can be inactivated by the λ recombinant protein phosphatase, hydrolysing phospholylated Ser, Thr, and Tyr residues. The effect of the recombinant phosphatase on dCK activity was completely inhibited by the addition of 1 mM Na-vanadate, the inhibitor of protein phosphatase activity (Fig. 5B). As a control, recombinant dCK was also treated with protein phosphatase in the presence (Fig. 5B) and absence (Fig. 5A) of 1 mM Na-vanadate without any effect on its activity. In order to exclude interfering protease

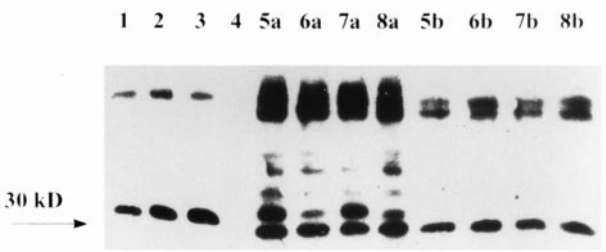

Fig. 4. Immunostaining of dCK protein before and after enzyme purification. Cells were incubated for 2 hr with or without different activators of dCK (5: control, 6: 1 μM CdA, 7: 6 μM APC, 8: 15 mM NaF). Samples used for SDS–PAGE, blotting, and immunostaining were derived either from the crude cell extracts (5a–8a) or from the peak fractions of column chromatography (5b–8b). Recombinant dCK was used as control (5, 20 and 40 ng in lanes 1–3).

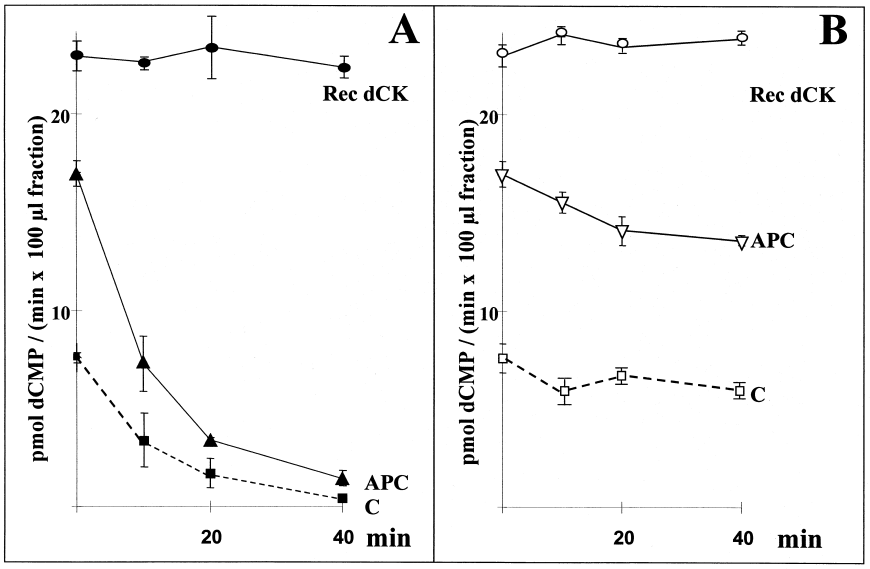

Fig. 5. Treatment of purified dCK with recombinant protein phosphatase. Lymphocytes were incubated in the presence (APC) or absence (C) of 6 μM APC for 2 hr. Cell extracts were purified as described in Fig. 3. (A) The purified dCK preparations (▲——▲: isolated from APC-treated cells, ■----■: isolated from control cells), as well as the recombinant dCK (●——●), were preincubated with λ recombinant protein phosphatase (4 U/μL) for different time periods, as indicated. dCK activity was measured after the protein phosphatase treatment, using a 10-fold dilution of the phosphatase-treated enzyme preparations, the standard dCK assay conditions, and 30-min incubation time. Activity of recombinant dCK is expressed as pmol dCMP/(min × μg protein). The figures illustrate mean values of three independent experiments; standard errors were <25%. (B) The same conditions as under (A), except that λ recombinant protein phosphatase (4 U/μL) activity was inhibited by 1 mM Na-vanadate during preincubation.

activity, immunostaining of these preparations was compared, and no degradation of dCK by protein phosphatase was seen during these time periods (data not shown).

4. Discussion

Increased dCK activity in cell cultures has relevance in both basic and clinical sciences in planning target-aimed combination chemotherapy. An interesting example of the benefit of high dCK activity resulting in increased sensitivity to 1-β-D-arabinofuranosylcytosine (araC) was shown by the elevated event-free survival rate of Down syndrome children with AML. The higher dCK activity is a possible consequence of chromosome 21 trisomy [28] as the dCK gene is localised on chromosome 21 [29]. Blast cells isolated from leukaemic children with Down syndrome had an elevated sensitivity to araC, as well as an unusually high dCK activity [28].

Our recent investigations were focused on the nature of agent that can activate dCK in normal and leukaemic lymphocytes and cell lines. Previously, CdA [17], as well as other deoxyribonucleoside analogues [18], were found to activate dCK in human lymphocytes and HL 60 cells. These data suggested that dCK was activated by its substrate analogues, explaining the clinical finding that CdA pretreatment increases the efficiency of subsequent araC treatment [30]. Beside the deoxynucleoside analogues, etoposide [18, 31] and NaF [19] were also effective in stimulating dCK. The common thread among these compounds was that they all inhibit DNA synthesis measured by ^{3}HdT incorporation. In this paper, we have investigated whether direct inhibition of DNA synthesis by APC leads to dCK activation. APC, a well-known inhibitor of DNA polymerases, was shown here to be the best stimulator of dCK (Table 1), suggesting the existence of a relationship between the inhibition of DNA synthesis and the activation of dCK. Recent results by Sargent *et al*. [32] are consistent with our observations, since APC pretreatment of blasts cells from AML patients increased their sensitivity to araC. This was probably caused by an increase in dCK activity. Increased accumulation of gemcitabine triphosphate, as the consequence of etoposide treatment, was shown in solid tumour cell lines, providing further evidence of the possible activation of dCK under certain conditions [33].

It was also shown here that the activated form of dCK survived partial purification, suggesting a conformational change in the protein structure rather than an interaction by a small ligand with non-covalent binding. As there was a high dilution of dCK during the purification procedure, any allosteric effect should be decreased during this step. On the other hand, purified dCK preparations were inactivated by protein phosphatase treatment. A series of controls were carried out to exclude any perturbation of the dCK assay conditions by recombinant protein phosphatase, namely by using its specific cofactor dependence as well as its inhibition by vanadate ions [34]. Furthermore, there was no protease contamination of the phosphatase preparations, since immunostaining showed no sign of degradation of phosphatase-treated dCK. Based on these data, activation and

inactivation of dCK through enzyme phosphorylation and dephosphorylation is very likely.

Purified dCK activation by enzyme phosphorylation was shown in a previous study, indicating that dCK could be a substrate *in vitro* for protein kinase C (PKC) [35]. Increased PKC activities were also shown as a response of HL 60 cell lines to araC treatment [36] or of ovarian cancer cells exposed to gemcitabine [37]. Potentiation of CdA treatment by briostatin, a PKC activator, was also shown to be accompanied by an elevated dCK level in a resistant chronic leukaemia cell line [38].

The data presented here suggest that inhibition of DNA synthesis activates dCK in normal and leukaemic peripheral human lymphocytes and in HL 60 cell lines. Up-regulation of dCK under inhibition of DNA replication could be surprising, since deoxythimidine kinase 1, another deoxynucleoside salvage enzyme, was shown to be down-regulated after treatment with drugs that affect DNA replication [39]. However, dNTP supply of DNA repair and membrane phospholipid synthesis [40] is possible through the action of dCK without dTK. Therefore, inhibition of DNA synthesis and induction of a "survival signal" might lead to activation of dCK.

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