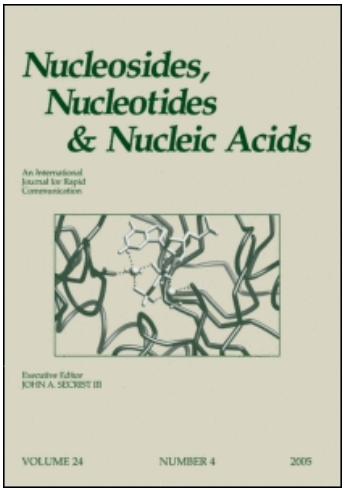

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Deoxycytidine Kinase is Reversibly Phosphorylated in Normal Human Lymphocytes

G. Keszler[a]; T. Spasokoukotskaja[a]; M. Sasvári-Székely[a]; S. Eriksson[b]; M. Staub[a]
[a] Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary [b] Department of Molecular Biosciences, Section of Veterinary Medical Biochemistry, SLU, the Biomedical Centre, Uppsala, Sweden

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DEOXYCYTIDINE KINASE IS REVERSIBLY PHOSPHORYLATED IN NORMAL HUMAN LYMPHOCYTES

G. Keszler, T. Spasokoukotskaja, and M. Sasvári-Székely □ *Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary*

S. Eriksson □ *Department of Molecular Biosciences, Section of Veterinary Medical Biochemistry, SLU, the Biomedical Centre, Uppsala, Sweden*

M. Staub □ *Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary*

□ *The activity of deoxycytidine kinase (dCK) has been shown to be enhanced upon genotoxic stress in human lymphocytes, and reversible phosphorylation of the enzyme has been implicated in the activation process. Here, we provide compelling evidence that dCK is a cytosolic phosphoprotein. Two-dimensional gel electrophoresis revealed that dCK has several differentially charged isoforms in cells. One-third of total cellular dCK was bound to a phosphoprotein-binding column irrespective of its activity levels, indicating that other mechanisms rather than phosphorylation alone might also be involved in the stimulation of enzyme activity. We excluded the possibility that activated dCK is translocated to the nucleus, but identified a dCK isoform of low abundance with a higher molecular weight in the nuclear fractions.*

Keywords Deoxycytidine kinase; Protein phosphorylation; Two-dimensional gel electrophoresis; Cell fractionation

INTRODUCTION

Apart from its physiological role in deoxynucleoside salvage pathways in lymphoid cells, sufficient activities of deoxycytidine kinase (dCK) are indispensable for the efficacy of nucleoside analogue-based chemotherapy.[1]

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Address correspondence to G. Keszler, Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, POB 260H-1444, Budapest, Hungary. E-mail: keszler@puskin.sote.hu

Recently, we and others have reported that dCK activity can be elevated profoundly upon genotoxic stress in both normal and leukemic human lymphocytes. This effect might have implications for developing tailored anticancer protocols.[2,3]

Several lines of indirect evidence indicated that dCK activation involved a phosphorylation-dependent step. Treatment of intact cells with calyculin A and okadaic acid, broad-spectrum inhibitors of serine/threonine-specific protein phosphatases, significantly increased dCK activities.[4,5] On the other hand, the dCK activity of cell extracts was abolished by λ protein phosphatase digestion.[6] Importantly, Smal et al.[7] demonstrated that recombinant, His-tagged dCK purified from HEK-293 cell extracts appeared as doublet bands on denaturing westeren blots, and the band with a slightly retarded mobility disappeared upon phosphatase treatment. Based on these observations, we wished to study the phosphorylation status of the endogenous dCK in human tonsillar lymphocytes.

MATERIALS AND METHODS

Isolation of human tonsillar lymphocytes, maintenance and treatment of primary cell cultures, preparation of whole-cell extracts, native and denaturing western blots were performed essentially as described in.[8] Aphidicolin and recombinant λ protein phosphatase were obtained from Sigma.

Two-dimensional gel electrophoresis of dCK samples immunoprecipitated from whole-cell extracts of control as well as of aphidicolin-treated cells was carried out using a Protean Isoelectric Focusing System (Bio-Rad Laboratories) with precast, immobilized pH gradient ampholyte strips (broad range, pH 3–10), followed by the second-dimension resolution on SDS-PAGE and blotting to PVDF membranes. Spots corresponding to dCK were visualized by denaturing western blot using a polyclonal dCK antibody recognizing the very C-terminal peptide of the enzyme.[9]

Isolation of the phosphorylated dCK pool was performed using the PhosphoProtein Purification Kit (Qiagen), according to the manufacturer's instructions. The flow-through and eluted fractions were concentrated with nanosep ultrafiltration columns and their dCK activities were determined with 10 μM ^{3}H-dCyd substrate as described in.[8]

Subcellular fractionation of tonsillar lymphocytes into nuclear and cytosolic fractions was performed as described by Hatzis et al.[9]

RESULTS AND DISCUSSION

In contrast to results obtained in an artificial overexpression system,[7] endogenous dCK has never been detected as doublet bands[3,4,8] (Figure 1,

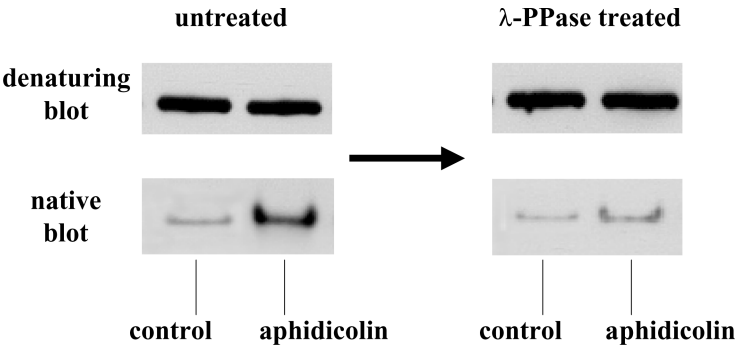

FIGURE 1 Decreased immunoreactivity of dCK upon λ protein phosphatase digestion. Of total protein 5 μg from control or aphidicolin-treated (2 hours) human lymphocytes was blotted either under denaturing (upper panels) or native (lower panels) conditions, prior to (left panels) or after (right panels) phosphatase treatments,[6] and probed with a highly specific dCK antibody.

left upper panel), not even in samples enriched by immunoprecipitation (data not shown). However, appearing as partially or totally split bands on denaturing western blots is not a general feature of phosphoproteins. Previously we reported that dCK activated by aphidicolin treatment of cells adopts a more open conformation resulting in enhanced native immunoreactivity.[8] As depicted in Figure 1, digestion of crude extracts of control and aphidicolin-treated cells with λ protein phosphatase dramatically decreased the immunoreactivity of dCK, strongly indicating that the conformational change was due to either direct phosphorylation of the enzyme, or to its interaction with a putative regulatory phosphoprotein partner.

Two-dimensional electrophoretic assays revealed that the total cellular dCK population comprises multiple isoforms with different isoelectric points (Figure 2). Early studies already identified 2 dCK polypeptides from human leukemic spleen with different pI values (5.5 and 5.7), implying that

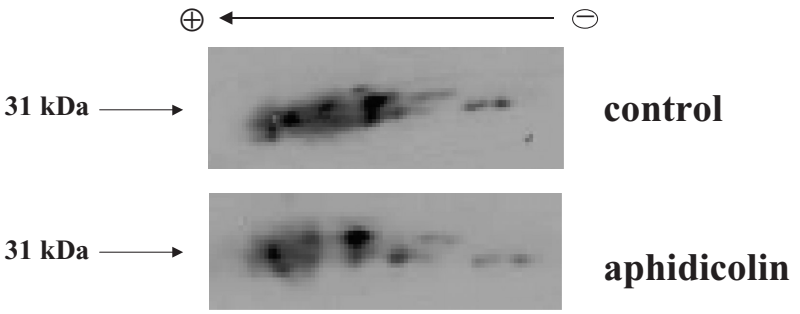

FIGURE 2 Two-dimensional gel electrophoresis and anti-dCK western blots from control and aphidicolin-treated cell extracts. See Materials and Methods for details.

the two subunits of the dCK dimer might have different structures.[10] In our case, however, 2 major and approximately 8 minor dots could be identified, raising a plausible possibility that dCK might be phosphorylated at multiple residues to different degrees, displaying various phosphorylation patterns. Surprisingly, however, the migration patterns of control and aphidicolin-stimulated samples essentially were identical, indicating that enzyme activation was not accompanied by gross alterations in the phosphorylation state of the enzyme.

These results were corroborated by phosphoprotein affinity chromatography. Approximately one third of total cellular dCK activity was retained on a phosphoprotein binding column (Qiagen) irrespectively of using crude extracts prepared either from control or from aphidicolin-treated lymphocytes.

Although deoxycytidine kinase was found to be localized in the cytosol, the enzyme harbors a putative nuclear localization signal that might direct it to the nucleus under certain conditions (e.g., during its activation).[9] To address this issue, control and aphidicolin-treated normal lymphocytes were fractionated into cytosolic and nuclear fractions, which were subsequently analyzed by western blotting. Figure 3 provides clear-cut evidence that dCK is not translocated to the nucleus upon its aphidicolin-induced activation. Interestingly, our antibody cross-reacted with a protein band of slightly higher molecular weight that could be detected exclusively in the nuclear fractions (Figure 3). Phosphatase treatment did not alter the electrophoretic mobility of that band, excluding the possibility that it might correspond to a hyperphosphorylated form of dCK (data not shown). It is

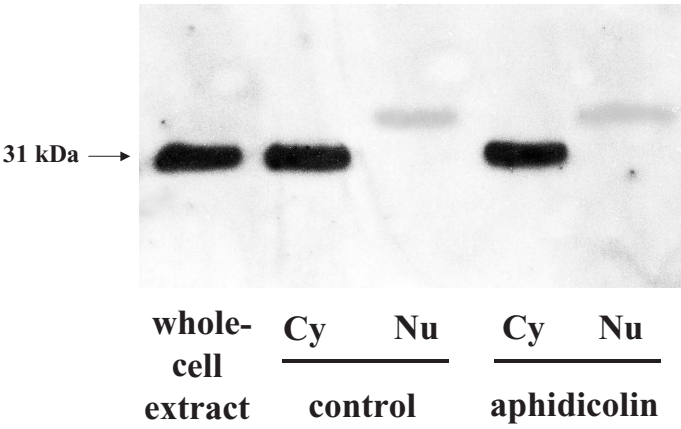

FIGURE 3 Activated dCK does not translocate to the nucleus. Cytosolic (Cy) and nuclear fractions (Nu) were prepared from cell cultures treated either with 2 μg/μl aphidicolin for 2 hours or left untreated (control). Whole-cell freeze-thaw extracts were prepared from control cells. All extracts were separated by SDS-PAGE and subjected to anti-dCK western blot.

tempting to speculate that it might represent an alternatively spliced nuclear isoform of dCK but this assumption needs further substantiation.

In summary, our data indicate that dCK is a cytosolic phosphoprotein, but its phosphorylation per se might not be sufficient for activation in normal human lymphocytes. Therefore, other mechanisms such as protein-protein interactions should also be involved in the activation process.

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