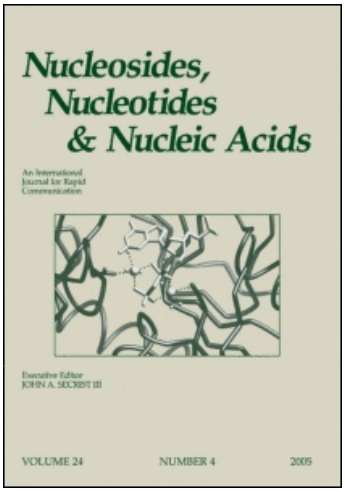

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INFLUENCE OF PHOSPHORYLATION OF THR-3, SER-11, AND SER-15 ON DEOXYCYTIDINE KINASE ACTIVITY AND STABILITY

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□ *Deoxycytidine kinase (dCK) is a key enzyme in the salvage of deoxyribonucleosides and in the activation of several anticancer and antiviral nucleoside analogues. We have recently shown that dCK is a phosphoprotein. Four in vivo phosphorylation sites were identified: Thr-3, Ser-11, Ser-15, and Ser-74. Site-directed mutagenesis demonstrated that phosphorylation of Ser-74, the major phosphorylated residue, strongly influences dCK activity in eucaryotic cells. Here, we show that phosphorylation of the three other sites, located in the N-terminal extremity of the protein, does not significantly modify dCK activity, but phosphorylation of Thr-3 could promote dCK stability.*

Keywords Deoxycytidine kinase; phosphorylation sites; site-directed mutagenesis; HEK 293T cells

INTRODUCTION

Deoxycytidine kinase (dCK) is a key enzyme in the salvage of deoxyribonucleosides and in the activation of numerous anticancer and antiviral nucleoside analogues.[1] Various genotoxic agents, such as etoposide, 2-chloro-2′-deoxyadenosine (CdA) and UV-C light, have been shown to increase dCK activity in leukemic cells without changing the level of dCK protein, suggesting that dCK activity could be regulated by post-translational modification.[2–4] We were the first to demonstrate that dCK, overexpressed in HEK (human embryonic kidney) 293T cells, is a phosphoprotein containing several phosphorylation sites: Thr-3, Ser-11, Ser-15, and Ser-74. Site-directed mutagenesis experiments demonstrated that Ser-74 phosphorylation is crucial for dCK activity in HEK 293T cells. Using a specific anti-pSer-74 antibody, we also detected phosphorylation of Ser-74 on endogenous dCK in leukemic cells. Moreover, we established that agents that enhance dCK activity increase the phosphorylation of this residue.[5,6] The purpose of the present study

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was to investigate the role of Thr-3, Ser-11, and Ser-15 phosphorylation on dCK activity.

MATERIALS AND METHODS

The coding region of the human dCK cDNA from leukemic lymphocytes was cloned and single mutations (T3A, T3E, S11A, S11E, S15A, S15E) were created by PCR using specific primers. The complete dCK coding region of all plasmids was sequenced on a CEQ2000 sequencer (Beckman Coulter Brea, CA, USA) to verify the newly introduced mutations and the absence of random mutation. HEK 293T cells, cultured as described in,[5] were transfected using the JetPEI procedure according to the manufacturer's instructions (PolyPlus transfection). Activity of dCK was measured in HEK 293T cell extracts, as reported previously,[5] using 5 mM ATP and 10 μM [5-^{3}H]-deoxycytidine (dCyd) or 50 μM [8-^{3}H]-2-chloro-2′-deoxyadenosine (CdA) as substrates. The protein content of samples was measured by the method of Bradford.[7] For immunoblot analyses, cell lysates (2 μg of protein) were subjected to SDS-PAGE in gel containing 12% acrylamide and transferred to low fluorescence PVDF immobilon-FL membranes (Millipore, Billerica, MA, USA). After transfer, membranes were blocked in Odyssey blocking buffer and probed overnight at 4°C with either anti-poly(His) (GE Healthcare) or anti-pSer-74 antibodies diluted (1/4000 and 1/1000, respectively) in Odyssey blocking buffer containing 0.1% Tween. After washing in PBS-T (Tween 0.1%), the membranes were incubated for 1 hour at room temperature with the appropriate fluorescent secondary antibody IRDye 800 or IRDye 680 (LI-COR Biosciences) diluted (1/10000) in Odyssey blocking buffer containing 0.1% Tween and 0.1% SDS. After washing, the membranes were scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences). Fluorescence intensities were used to quantify the expression of dCK and its phosphorylation on Ser-74.

RESULTS

To test whether phosphorylation of Thr-3, Ser-11 and Ser-15 was important for dCK activity, HEK 293T cells were transiently transfected with various mutated His-tagged dCK (T3A, T3E, S11A, S11E, S15A, S15E), in which the phosphorylation sites were replaced through site-directed mutagenesis by Ala (A) to abolish, or by Glu (E) to mimic their phosphorylation. After 48 hours, dCK activity was measured in cell lysates with a pyrimidine (dCyd) (Figure 1A) or a purine (CdA) (Figure 1B) nucleoside as substrate and dCK protein level was assessed by fluorometric quantification using anti-poly(His) antibody (Figure 1C and 1D). None of the introduced mutations significantly modified normalized dCK activity, neither with dCyd nor with CdA. However, as indicated by the data presented in the lower part of

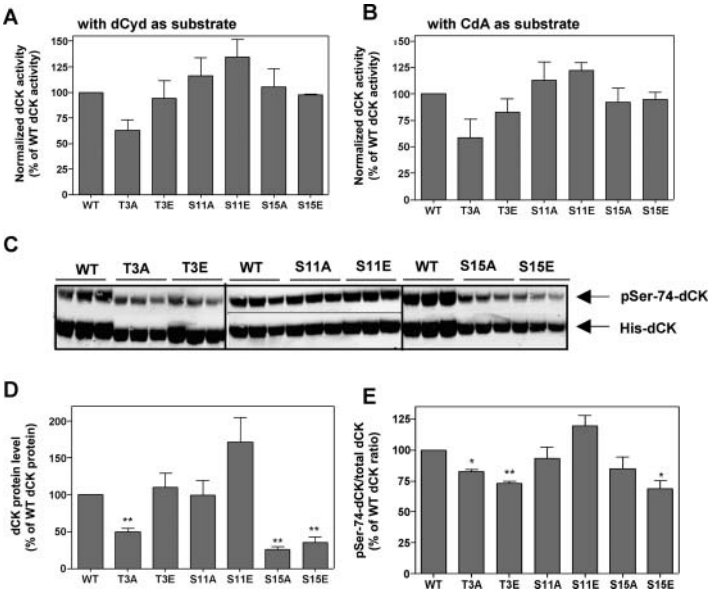

FIGURE 1 Effect of mutation of Thr-3, Ser-11, and Ser-15 on dCK activity, dCK protein level, and phosphorylation of dCK on Ser-74. dCK activity was measured with dCyd (A) or CdA (B) in lysates of HEK 293T cells transfected with wild-type (WT) or mutated dCK, as indicated. Activities were normalized to dCK protein amount analyzed with the anti-His antibody (C, lower part, and D), as explained in the text. Analysis of Ser-74 phosphorylation was done with the anti-pSer-74 antibody, as illustrated in C (upper part), and pSer-74-dCK/total dCK ratios were calculated (E). Results illustrated in A, B, D, E are means ± SEM of three independent experiments, in which duplicate or triplicate were performed. Results shown in C are representative from experiments illustrated in D and E. $^{*}P < 0.05$; $^{**}P < 0.01$.

Figure 1C and confirmed in two other independent experiments, the T3A, S15A, and S15E mutated dCK were less expressed at the protein level than wild-type (WT) dCK (Figure 1D).

To complete this study, we sought to analyze whether phosphorylation of Thr-3, Ser-11, and Ser-15 might influence Ser-74 phosphorylation. Therefore, HEK 293T cell lysates were probed with the anti-pSer-74 antibody (upper part of Figure 1C) and pSer-74-dCK/total dCK ratios of the various mutants were compared with that of WT dCK (Figure 1E). A slight, but significant, reduction of Ser-74 phosphorylation was observed for the T3A, T3E, and S15E dCK mutants.

DISCUSSION

Phosphorylation of terminal residues of some proteins can promote conformational changes leading to activation or inhibition of enzymes,[8] alter the surface properties of the protein that may affect self association or association of the protein with other molecules,[9] and finally influence proteasomal degradation.[10,11] Therefore, it was important to investigate the role of Thr-3, Ser-11, and Ser-15 on the activity and stability of dCK.

We did not find a significant change in dCK activity by replacing Thr-3, Ser-11, and Ser-15 by Ala or Glu, indicating that phosphorylation of these residues does not play a role in the control of dCK activity. However, we noted a significant decrease in dCK protein level of three mutants: T3A, S15A, and S15E. Moreover, a significant difference was found between the protein levels of the T3A and T3E mutants ($P = 0.05$), suggesting that phosphorylation of Thr-3 could stabilize dCK. Conversely, no significant difference was observed between the protein levels of the S15A and S15E mutants ($P = 0.25$), indicating that the reduced protein level of these two mutants is probably due to the replacement of Ser by another residue, which may induce an unfavourable structural change of the protein.

Besides a direct role in the control of dCK activity, phosphorylation of Thr-3, Ser-11, or Ser-15 could be required for subsequent phosphorylation of dCK on Ser-74 by a phosphate-directed kinase. However, analysis of the normalized phosphorylation level of Ser-74 in the various constructed mutants does not support this hypothesis: some changes were significant, but very small, and without significant effect on dCK activity. Further studies are planned to investigate whether phosphorylation of Thr-3, Ser-11, and Ser-15 might be involved in other types of regulation, like intracellular localization or interaction with other protein partners.

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