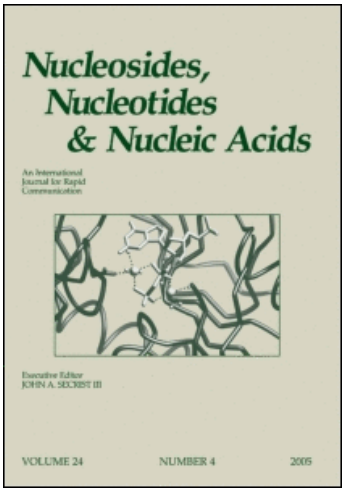

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Identification of Phosphorylation Sites on Human Deoxycytidine Kinase After Overexpression in Eucaryotic Cells

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IDENTIFICATION OF PHOSPHORYLATION SITES ON HUMAN DEOXYCYTIDINE KINASE AFTER OVEREXPRESSION IN EUCARYOTIC CELLS

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□ *Compelling evidence suggests that deoxycytidine kinase (dCK), a key enzyme in the salvage of deoxyribonucleosides and in the activation of clinically relevant nucleoside analogues, can be regulated by reversible phosphorylation. In this study, we show that dCK overexpressed in HEK-293T cells was labelled after incubation of the cells with [^{32}P]orthophosphate. Tandem mass spectrometry allowed the identification of 4 in vivo phosphorylation sites, Thr3, Ser11, Ser15, and Ser74. These results provide the first evidence that dCK is constitutively multiphosphorylated in intact cells. In addition, site-directed mutagenesis demonstrated that phosphorylation of Ser74, the major in vivo phosphorylation site, is crucial for dCK activity.*

Keywords Deoxycytidine kinase; Protein phosphorylation; Tandem mass spectrometry

INTRODUCTION

Deoxycytidine kinase (dCK) catalyzes the phosphorylation of deoxycytidine, deoxyadenosine, and deoxyguanosine, playing a major role in

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the deoxynucleoside salvage pathway. Moreover, dCK phosphorylates and activates numerous nucleoside analogues used against leukemias, solid tumors, and viral infections. Several genotoxic agents, including etoposide, aphidicolin, UV-light, γ-irradiation, and even the nucleoside analogue CdA, have been shown to induce dCK activation without changing the level of dCK protein.[1–4] Moreover, treatment of lysates from normal or leukemic cells with λ protein phosphatase lead to a decrease of dCK activity.[1,4] Taken together these results indicated that dCK could be regulated by post-translational modification, most probably by reversible protein phosphorylation. To verify this hypothesis, eucaryotic cells (HEK 293T, human embryonic kidney cells), overexpressing dCK as a His-tag fusion protein, were incubated with [^{32}P]orthophosphate and dCK labelling was analyzed.

MATERIALS AND METHODS

The coding region of the human dCK cDNA from leukemic lymphocytes was cloned as previously described.[5] Recombinant dCK with a N-terminal polyhistidine tag was overexpressed in HEK 293T cells using the jetPEI procedure according to the manufacturer's instructions (PolyPlus transfection, Illkrich, France). Mutations (S74A or S74E) were created by PCR using specific primers. The complete dCK coding region of all plasmids was sequenced on a CEQ2000 sequencer (Beckman Coulter, Fullerton, CA, USA) to verify the newly introduced mutations and the absence of random mutation. For in vivo labelling experiments, HEK 293T cells overexpressing dCK were incubated in phosphate-free DMEN containing [^{32}P]orthophosphate (4 mCi/dish, 670 μCi/ml) for 3.5 hours. Recombinant dCK was purified by affinity chromatography using an agarose-cobalt resin (Clontech/BD Biosciences, Palo Alto, CA, USA), concentrated by ultrafiltration and subjected to SDS-PAGE/autoradiography. Bands corresponding to dCK were cut from the gel, chopped into 1 mm cubes and digested with trypsin. Labelled tryptic peptides were separated by reverse–phase HPLC and the radioactivity of each fraction was counted by Cerenkov radiation. Radioactive peaks were analysed by nanoelectrospray ionization tandem mass spectrometry (nano-ESI-MS/MS), as described in Woods et al.[6] DCK activity was determined with 10 μM [5-^{3}H]deoxycytidine as the substrate, as previously described.[4] Western blotting was performed with monoclonal antibodies against poly-His (1/2000) in PBS-T (0.1%) and 5% powder milk.

RESULTS

To know whether dCK was constitutively phosphorylated, we incubated HEK-293T cells overexpressing dCK in the presence of

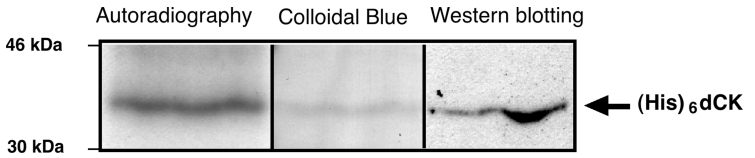

FIGURE 1 Phosphorylation of dCK in HEK 293T cells. HEK 293T cells, overexpressing dCK, were incubated with [^{32}P]orthophosphate for 3.5 hours. dCK was purified from cell extracts by affinity chromatography, concentrated by ultrafiltration, and subjected to SDS-PAGE after staining the gel with Colloidal Blue. PolyHis-tagged dCK was detected by immunoblotting under the conditions used for ^{32}P-incorporation. (From Smal et al.[8])

[^{32}P]orthophosphate. dCK was purified by affinity chromatography and subjected to SDS-PAGE and autoradiography (Figure 1). A labelled band of 34 kDa was detected, corresponding to the major band revealed by colloidal blue and to the band recognized by the monoclonal anti-polyHis antibody, indicating that the labelled band was indeed recombinant dCK. This molecular mass of 34 kDa is somewhat greater than that of 30.5 kDa established for the subunit of dCK,[7] but corresponds to the calculated molecular mass of the polyhistidine-tagged recombinant fusion protein.

Radiolabelled dCK, purified by affinity chromatography, was then digested with trypsin, and peptides were separated by reverse-phase HPLC. Fractions were counted by emission of Cerenkov radiation. Several radioactive peaks were detected, indicating the presence of multiple phosphorylation sites in the recombinant protein (Figure 2). Except Peak I (the flow-trough fraction), each radioactive peak was screened for phosphopeptides by neutral loss of H_3PO_4 (98 Da) using nano-ESI-MS/MS, as described in Woods et al.[6] This technique allowed the identification of 4 phosphorylated residues, as indicated in Table 1. In Peak IV, no phosphopeptide was detected. The relative labelling of the HPLC peaks was calculated in three

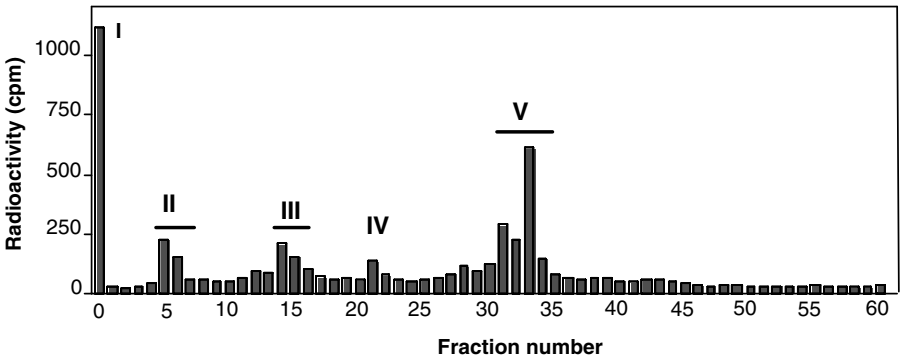

FIGURE 2 HPLC profile of ^{32}P-peptides obtained by tryptic digestion of dCK from transfected HEK 293T cells. ^{32}P-dCK, obtained as described in Figure 1, was excised from the gel and digested with trypsin. Labelled peptides were separated by reverse-phase narrow-bore HPLC in a linear acetonitrile gradient. Fractions were counted by Cerenkov radiation. (From Smal et al.[8])

TABLE 1 Phosphorylated dCK Residues Determined by Nano-ESI-MS/MS

Peak	Phosphorylated residue	% of total ^{32}P-incorporated
I		22.3 ± 8.9
II	Thr3	10.9 ± 5.7
III	Ser11, Ser15	9.9 ± 1.7
IV	ND	10.3 ± 3.7
V	Ser74	44.6 ± 6.2

Labelled HPLC peaks (Figure 2) were analyzed by nano-ESI-MS/MS. Phosphopeptides were identified in MS^2 mode by loss of 98 Da upon collision-induced dissociation, and the phosphorylated residue was further identified by fragmentation in MS^3 mode. The relative labelling of the HPLC peaks was calculated in 3 separate experiments. Percentage of total ^{32}P-incorporation are means ± SEM ($n = 3$). ND: not determined.

separate experiments. Ser74 appeared as the major in vivo phosphorylation site.

Site-directed mutagenesis experiments were performed to test the importance of Ser74 phosphorylation for dCK activity. HEK 293T cells were transfected with mutated His-tagged dCK. Ser74 was replaced by alanine to abolish phosphorylation at this locus (S74A mutant) or by glutamate to mimic its phosphorylation (S74E mutant). The S74E mutation did not significantly modify dCK activity (Figure 3), whereas the S74A mutation markedly decreased activity (by 94.1 ± 1.7%), suggesting that phosphorylation of Ser74 is crucial for dCK activity. Mutations did not affect the Km of dCK for deoxycytidine, but only the Vmax (results not shown).

DISCUSSION

This study provides a definitive proof that dCK is a phosphoprotein in eucaryotic cells. The enzyme contains at least 4 phosphorylation sites, but

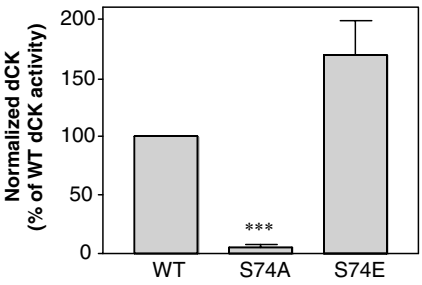

FIGURE 3 Effect of mutation of Ser74 on dCK activity. DCK activity was measured in lysates of HEK293T cells transfected with vector encoding wild-type dCK (WT) or the S74A or S74E mutants. Activities were normalized to dCK protein amount assessed by densitometric quantification using anti-polyHis antibody. The results are means ± SEM of three independent experiments. WT dCK activities were set at 100%. Significance relative to the WT dCK: ***$P<0.001$. (From Smal et al.[8])

other phosphorylations sites probably exist as suggested by the labelling of peaks I and IV in the HPLC profile (Figure 2), in which the phosphorylated residue was not yet identified. The 3-dimensional structure of human dCK was recently solved by Sabini et al.[7] The enzyme is homodimeric with a fold similar to that described for the *Drosophila melanogaster* deoxynucleoside kinase. Each monomer consists of ten α-helices surrounding a 5-stranded parallel β-sheet. The N-terminal extremity of dCK (residues 1–19), which contains 3 of the 4 phosphorylation sites identified, is flexible and its structure could not be solved. However, this region predicted to lie outside the protein core. Ser74 is located in a 15-residue mobile insert. Thus, the 4 phosphorylation sites we identified are located in flexible loops at the surface of the protein and would be accessible to protein kinases. As indicated by site-directed mutagenesis experiments, phosphorylation of Ser74, the major in vivo phosphorylation site, is critical for the catalytic activity of dCK, suggesting that agents known to enhance dCK activity in lymphocytes might act by increasing the phosphorylation of Ser74. This hypothesis was confirmed in a further study.[8]

Recently, Keszler et al.[9] suggested that activation of dCK, subsequent to a posttranslational modification, induced a more open conformation of the enzyme. We propose that phosphorylation of Ser74, which is located far from the active site of dCK, might induce long-range conformational changes in dCK and promote such open conformation.

The protein kinases responsible for Ser74 phosphorylation remain to be identified. It has been reported that PKCα can phosphorylate dCK purified from leukemic blasts and increase the enzyme activity.[10] These results were, however, not reproduced with a human recombinant dCK.[11]

The identification of phosphorylation sites on dCK, and particularly of Ser74, could have implications for improving the activation of nucleoside analogues used in anti-cancer and anti-viral chemotherapy, or for the design of more active dCK mutants for suicide-gene therapy.

REFERENCES

1. Csapo, Z.; Sasvari-Szekely, M.; Spasokoukotskaja, T.; Talianidis, I.; Eriksson, S.; Staub, M. Activation of deoxycytidine kinase by inhibition of DNA synthesis in human lymphocytes. *Biochem. Pharmacol.* **2001**, 61, 191–197.
2. Csapo, Z.; Keszler, G.; Safrany, G.; Spasokoukotskaja, T.; Talianidis, I.; Staub, M.; Sasvari-Szekely, M. Activation of deoxycytidine kinase by gamma-irradiation and inactivation by hyperosmotic shock in human lymphocytes. *Biochem. Pharmacol.* **2003**, 65, 2031–2039.
3. Van Den Neste, E.; Cardoen, S.; Delacauw, A.; Frankard, J.; Ferrant, A.; Van den Berghe, G.; Bontemps, F. Activation of deoxycytidine kinase by UV-C-irradiation in chronic lymphocytic leukemia B-lymphocytes. *Biochem. Pharmacol.* **2003**, 65, 573–580.
4. Smal, C.; Cardoen, S.; Bertrand, L.; Delacauw, A.; Ferrant, A.; Van Den Berghe, G.; Van Den Neste, E.; Bontemps, F. Activation of deoxycytidine kinase by protein kinase inhibitors and okadaic acid in leukemic cells. *Biochem. Pharmacol.* **2004**, 68, 95–103.

5. Smal, C.; Bertrand, L.; Van Den Neste, E.; Cardoen, S.; Veiga-da-Cunha, M.; Marie, S.; Race, V.; Ferrant, A.; Van Den Berghe, G.; Bontemps, F. New evidences for a regulation of deoxycytidine kinase activity by reversible phosphorylation. *Nucleosides Nucleotides Nucleic Acids* **2004**, 23, 1363–1365.
6. Woods, A.; Vertommen, D.; Neumann, D.; Turk, R.; Bayliss, J.; Schlattner, U.; Wallimann, T.; Carling, D.; Rider, M.H. Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. *J. Biol. Chem.* **2003**, 278, 28434–28442.
7. Sabini, E.; Ort, S.; Monnerjahn, C.; Konrad, M.; Lavie, A. Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. *Nat. Struct. Biol.* **2003**, 10, 513–519.
8. Smal, C.; Vertommen, D.; Bertrand, L.; Ntamashimikiro, S.; Rider, M.H.; Van Den Neste, E.; Bontemps, F. Identification of in vivo phophorylation sites on human deoxycytidine kinase. Role of Ser-74 in the control of enzyme activity. *J. Biol. Chem.* **2006**, 281, 4887–4893.
9. Keszler, G.; Spasokoukotskaja, T.; Csapo, Z.; Talianidis, I.; Eriksson, S.; Staub, M.; Sasvari-Szekely, M. Activation of deoxycytidine kinase in lymphocytes is calcium dependent and involves a conformational change detectable by native immunostaining. *Biochem. Pharmacol.* **2004**, 67, 947–955.
10. Wang, L.M.; Kucera, G.L. Deoxycytidine kinase is phosphorylated in vitro by protein kinase C alpha. *Biochim. Biophys. Acta* **1994**, 1224, 161–167.
11. Spasokoukotskaja, T.; Csapo, Z.; Sasvari-Szekely, M.; Virga, S.; Talianidis, I.; Eriksson, S.; Staub, M. Effect of phosphorylation on deoxycytidine kinase activity. *Adv. Exp. Med. Biol.* **2000**, 486, 281–285.