Identification of in Vivo Phosphorylation Sites on Human Deoxycytidine Kinase

ROLE OF SER-74 IN THE CONTROL OF ENZYME ACTIVITY

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Deoxycytidine kinase (dCK) catalyzes the rate-limiting step of the deoxyribonucleoside salvage pathway in mammalian cells and plays a key role in the activation of numerous nucleoside analogues used in anti-cancer and antiviral chemotherapy. Although compelling evidence indicated that dCK activity might be regulated by phosphorylation/dephosphorylation, direct demonstration was lacking. Here we showed that dCK overexpressed in HEK 293T cells was labeled after incubating the cells with [32P]orthophosphate. Sorbitol, which was reported to decrease dCK activity, also decreased the labeling of dCK. These results indicated that dCK may exist as a phosphoprotein in vivo and that its activity can be correlated with its phosphorylation level. After purification of [32P]-labeled dCK, digestion by trypsin, and analysis of the radioactive peptides by tandem mass spectrometry, the following four in vivo phosphorylation sites were identified: Thr-3, Ser-11, Ser-15, and Ser-74, the latter being the major phosphorylation site. Site-directed mutagenesis and use of an anti-phospho-Ser-74 antibody demonstrated that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential. Phosphorylation of Ser-74 was also detected on endogenous dCK in lymphoid cells, in which the Ser-74 phosphorylation state was increased by agents that enhanced dCK activity. Our study provided direct evidence that dCK activity can be controlled by phosphorylation in intact cells and highlights the importance of Ser-74 for dCK activity.

Deoxycytidine kinase (dCK; EC 2.7.1.74) catalyzes the phosphorylation of deoxycytidine, deoxyguanosine, and deoxyadenosine, with ATP or UTP as phosphoryl donor. This reaction is the rate-limiting step of the deoxyribonucleoside salvage pathway that supplies cells with precursors of DNA as an alternative to de novo synthesis (1). In addition, dCK initiates the activation of several chemotherapeutic nucleoside analogues, such as 1-β-D-arabinofuranosylcytosine (cytarabine), 9-β-D-arabinosyl-2-fluoroaradenine (fludarabine), and 2-chloro-2'-deoxyadenosine (CdA, cladribine), commonly used in the treatment of hematological malignancies, and 2',2'-difluorodeoxycytidine (gemcitabine), active against solid malignant tumors (2–4). The anti-human immunodeficiency virus drugs 2',3'-dideoxycytidine (zalcitabine) and 2'-deoxy-3'-thiacytidine (lamivudine) are also phosphorylated by dCK (5). Phosphorylation of these inactive pro-drugs by dCK is a prerequisite for their pharmacological action, as demonstrated by the resistance of cells lacking dCK activity to nucleoside analogues (6–9). Moreover, a number of in vitro and in vivo studies indicated a positive correlation between dCK activity and nucleoside analogue sensitivity (10–15). The enzyme is preferentially expressed in lymphoid cells (1), which explains the clinical success of nucleoside analogues against lymphoproliferative disorders, such as hairy cell leukemia and B-cell chronic lymphocytic leukemia (16, 17).

Because dCK plays an essential role in the therapeutic efficacy of nucleoside analogues, identification of mechanisms that control dCK activity is of particular interest. In recent years, several genotoxic agents, including DNA polymerase and topoisomerase II inhibitors, UV light, γ-irradiation, and nucleoside analogues such as CdA, have been shown to activate dCK in human normal or leukemic lymphocytes (18–23). Activation of dCK by these agents could not be explained by allosteric effects or by a change in dCK protein levels, suggesting that dCK activity might be regulated by reversible covalent modification, e.g. via phosphorylation/dephosphorylation. Accordingly, the activity of dCK in extracts of normal or leukemic lymphocytes was markedly decreased on treatment with λ-protein phosphatase (23) or protein phosphatase 2A (24). Also, further studies indicated that dCK might be dephosphorylated in vivo by protein phosphatase 2A (24). Moreover, dCK activity was surprisingly enhanced by several cell-permeable protein kinase inhibitors in various types of leukemic cells (24) and decreased by hyperosmotic stress, known to induce extensive changes in several cell signaling pathways (23).

Despite all the evidence suggesting that dCK is regulated by reversible phosphorylation, direct demonstration was lacking. To verify that dCK is indeed phosphorylated, human dCK was expressed in human embryonic kidney (HEK) 293T cells as a His-tagged fusion protein. Following incubation of the cells with [32P]orthophosphate and purification of dCK, four phosphorylation sites were identified by mass spectrometric analysis of tryptic peptides. Phosphorylation of Ser-74, the most [32P]-labeled site in vivo, was found to be crucial for dCK activity in HEK 293T cells. In addition, data obtained in CCRF-CEM cells showed that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential. Phosphorylation of Ser-74 was also detected on endogenous dCK in lymphoid cells, in which the Ser-74 phosphorylation state was increased by agents that enhanced dCK activity. Our study provided direct evidence that dCK activity can be controlled by phosphorylation in intact cells and highlights the importance of Ser-74 for dCK activity.
Control of dCK Activity by Ser-74 Phosphorylation

phosphorylation could also be important for dCK activity in leukemic cells.

EXPERIMENTAL PROCEDURES

Materials—Sequencing-grade trypsin was purchased from Promega. HPLC-grade solvents and acids were from Acros Organics. Phosphate-free DMEM, pEF6/His expression vector, and T4 DNA ligase were from Invitrogen. Primers for cloning and site-directed mutagenesis were from Eurogentec (Ougrée, Belgium), Reverse transcriptase, T4 polynucleotide kinase, and Tag polymerase were from Fermentas (St. Leon-Rot, Germany). Pwo polymerase and restriction enzymes were from Roche Applied Science. Other chemicals, materials, and reagents were from Sigma, Calbiochem, or Bio-Rad.

Transfection, Illkirch, France) according to the manufacturer’s instructions. The complete dCK coding region of all plasmids was sequenced on a CEQ2000 sequencer (Beckman Coulter) to verify the newly introduced mutations and the absence of random mutations. The dCK cDNA was then subcloned into the eukaryotic pEF6/His vector between the BamH I and Xba I restriction sites for expression of normal or mutated polyhistidine fusion proteins with a His-tag at the N-terminal position.

Transfection and Purification of Recombinant dCK—For transient transfections, 1.5 × 106 HEK 293T cells were plated in 8.5-cm dishes and transfected the following day by the jetPEI procedure (PolyPlus Transfection, Illkirch, France) according to the manufacturer’s instructions and by using 8 µg of plasmid DNA. After 48 h, cells were washed with cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (buffer A) containing 50 mM HEPES, pH 7.5, 50 mM NaF, 1 mM K2HPO4, 0.1% (w/v) β-mercaptoethanol, 5 mM β-glycerophosphate, 5 mM Na3P04, 1% (w/v) Triton X-100, protease inhibitors (1 µM p-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μg/ml leupeptin, and antipain), and 1 mM sodium orthovanadate. Cells were lysed by one cycle of freeze-thawing. Cell lysates were centrifuged at 16,100 × g for 10 min, and supernatants were loaded on a Talon (Co2+) affinity column (Clontech).

After three washings with buffer B containing 50 mM HEPES, pH 7.5, 0.1% (w/v) β-mercaptoethanol, protease inhibitors, and 300 mM NaCl, followed by two washings with buffer B supplemented with 10 mM imidazole, recombinant dCK was eluted with buffer B devoid of NaCl but containing 150 mM imidazole. Depending on the experiment, the purified protein was stored at −80 °C in the presence of 20% glycerol, concentrated by ultrafiltration, precipitated with chloroform/methanol (25), or boiled in Laemmli SDS-PAGE buffer.

Protein Phosphatase Treatment of Cell Extracts—For dephosphorylation experiments, cell lysis was carried out in buffer A devoid of protein phosphatase inhibitors. Samples containing ~15 µg of cell protein were incubated at 30 °C with or without λ-protein phosphatase, as described previously (24). After 1 h, 10-µl aliquots were taken for the measurement of dCK activity. Western blotting with anti-poly(His) antibody was also performed on 1 µg of cell protein extract.

dCK Assay—HEK 293T cell extracts were prepared as described above. Extraction of CCRF-CEM cells and dCK assay was performed as reported previously (24), using 4–40 µg of cell extract protein or 20–200 ng of purified dCK for the measure of activity. Except for determining the Km value for deoxycytidine, dCK activity was measured under Vmax conditions, with 10 µM deoxycytidine and 5 mM ATP in the assays. The protein content of samples was measured by the method of Bradford (26) by using bovine serum albumin as standard.

Antibodies and Immunoblotting—Anti-dCK antibody raised against the C-terminal peptide of human dCK (amino acids 246–260) was generated by Eurogentec, according to the procedure of Hatzip et al. (27). Anti-poly(His) monoclonal antibody was from GE Healthcare. Anti-phospho-Ser-74 antibody was raised in New Zealand White rabbits injected with a synthetic phosphopeptide corresponding to amino acids 68–79 of human dCK with an N-terminal cysteine (CEELT-MpsQKNGG), for coupling to keyhole limpet hemocyanin.

For immunoblot analysis, HEK 293T cell lysates (1–5 µg) or purified dCK (50–100 ng) were subjected to SDS-PAGE in gels containing 12% (w/v) acrylamide and transferred to Hybond C-extra membranes (GE Healthcare, Roosendaal, The Netherlands). Membranes were blocked in PBS with 5% (w/v) fat-free milk powder and probed with either anti-poly(His) monoclonal antibody (1/2000), anti-phospho-Ser-74 antibody (1/1000), or anti-dCK antibody (1/1000) in PBS-T (Tween 0.1%, w/v) for 1 h at room temperature or overnight at 4 °C. After extensive washing in PBS-T, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horse-radish peroxidase (1/10,000). After further extensive washing in PBS-T, the blots were developed using enhanced chemiluminescence (GE Healthcare). Quantification of dCK expression or phosphorylation was carried out by using National Institutes of Health image software.

In Vivo Labeling with [32P]Orthophosphate—HEK 293T cells were cultured and transfected as described above. Two days after transfection, the cells were washed with phosphate-free DMEM and incubated for 3.5 h with the same medium containing [32P]orthophosphate (carrier-free; GE Healthcare) (4 µCi/dish, 670 µCi/ml). Four dishes were used for each experiment. Labeling was stopped by washing the cells twice with ice-cold PBS. Cells were lysed in 0.8 ml of buffer A. dCK was purified from cell extracts by affinity chromatography as described above, concentrated by ultrafiltration, and subjected to SDS-PAGE/autoradiography. Alternatively, dCK was immunoprecipitated from cell extracts with anti-poly(His) antibody coupled to protein A-Sepharose (GE Healthcare). Immune complexes were washed six times with 1 ml of buffer A containing 0.5 M NaCl, and three times with 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 0.1% (w/v) β-mercaptoethanol. The beads were mixed with Laemmli buffer, boiled, and subjected to SDS-PAGE/autoradiography.

Phosphorylation Site Identification by Tandem Mass Spectrometry—For in vivo phosphorylation site analysis, HEK 293T cells were labeled as described above, except that cells were preincubated for 1 h with 0.5 M sorbitol and washed before labeling and that the latter was performed in the presence of 0.5 µM okadaic acid. After purification by affinity chromatography, SDS-PAGE, and Colloidal Blue staining, bands corresponding to dCK were cut from the gel and chopped into 1-mm cubes. The gel pieces were washed, reduced with dithiothreitol, and treated with iodoacetamide prior the tryptic digestion and a two-step extraction of peptides (28). The yield of radioactivity in the two-step extraction was about 80% as determined by 32P counting by Cerenkov radiation. Labeled tryptic peptides obtained from 4 dishes of 32P-labeled HEK 293T cells were then mixed with
peptides from 10 dishes of unlabeled HEK 293T cells using exactly the same protocol. The combined extracts were concentrated to about 20 ūl in a SpeedVac® concentrator, and trifluoroacetic acid was added to a concentration of 0.1% (v/v). Peptides were separated by reverse-phase narrow-bore HPLC at a flow rate of 200 ūl/min (28). The radioactivity of each fraction was measured by Cerenkov counting. Radioactive peaks were concentrated to about 10 ūl, mixed with 0.1% (v/v) trifluoroacetic acid, desalted using a Ziptip C18 pipette tip (Millipore), eluted in 50% (v/v) acetonitrile, 0.3% (v/v) acetic acid, and analyzed by nanoelectrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) in an LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Spectra were taken in full MS and zoom scan mode to determine parent ion monoisotopic masses and their charge states. The source voltage was set at 0.8 kV, and the collision-induced dissociation energy was adjusted to the minimum needed for fragmentation. Phosphopeptides were identified in MS² mode by the loss of H₃PO₄, (98 Da) under low collision-induced dissociation energy, and the phosphorylated residue was pinpointed in MS³ mode.

Data Analysis—All experiments have been repeated two or more times. The results of experiments repeated at least three times are given as the means ± S.E. Significance was estimated by the paired two-tailed Student’s t test.

RESULTS

Properties of Human dCK Overexpressed in HEK 293T Cells—The activity of dCK measured after overexpression as a His tag fusion protein in HEK 293T cells was 500–1000-fold higher than in nontransfected cells. A one-step purification of (His)₆dCK by affinity chromatography gave a major 34-kDa band in Coomassie Blue-stained gels that was recognized following immunoblotting with anti-poly(His) antibody (data not shown). This molecular mass, somewhat greater than that of 30.5 kDa established by Chottiner et al. (29) for the dCK subunit, is in agreement with the calculated molecular mass of the polyhistidine-tagged recombinant fusion protein (34.3 kDa). Size exclusion chromatography (Sephacryl S-200) gave a single peak of dCK activity eluting with a molecular mass of 68 kDa, indicating that the active form of recombinant dCK is a dimer (result not shown), as reported for the native enzyme (30). The Kₘ value of purified recombinant (His)₆dCK for deoxyxycytidine, with 5 mM ATP as co-substrate, was 0.16 ± 0.01 mM (n = 3), in agreement with values reported previously for dCK expressed in bacteria (31).

Extracts of HEK 293T cells were incubated with A-protein phospha-
tase, which led to a marked (∼85%) decrease in dCK activity (Fig. 1A), as observed previously for the native enzyme from normal (21) or leukemia human lymphocytes (24). Moreover, this drop in activity was accompanied by a slight increase in electrophoretic migration of the recombinant protein on SDS-polyacrylamide gel (Fig. 1B), as often observed for nonphosphorylated versus phosphorylated proteins. These results indicated that the activity of dCK expressed in eukaryotic cells could be regulated by phosphorylation/dephosphorylation.

In Vivo Phosphorylation of dCK—To verify that dCK was phosphorylated in vivo, HEK 293T cells expressing recombinant dCK were incubated with [³²P]orthophosphate for 3.5 h. dCK was purified from cell extracts by affinity chromatography, concentrated by ultrafiltration, and subjected to SDS-PAGE and autoradiography after staining the gels with colloidal blue. Poly(His)-tagged dCK was detected by immunoblotting under the conditions used for [³²P] incorporation. One representative experiment is shown.

FIGURE 1. Dephosphorylation of recombinant dCK decreases activity and increases electrophoretic migration. A, activity of recombinant (His)₆dCK measured in HEK 293T cell lysates, treated with or without A-protein phosphatase (λ-PP), for 1 h. The results are means ± S.E. for three experiments. B, dCK analyzed by Western blot with anti-poly(His) antibody of individual lysates used in the experiments shown in A. A slight retardation in the mobility of the dCK band is seen in the three lysates that had not been treated with A-protein phosphatase.
detected, indicating the presence of multiple phosphorylation sites in the recombinant protein (Fig. 4). Five labeled peaks (I to V) were observed, with peak I appearing in the flow-through fraction. Except for peak I, each radioactive peak was screened for phosphopeptides by neutral loss of H3PO4 (98 Da) by nano-ESI-MS/MS. Peak II contained a phosphopeptide consistent with a single phosphate addition to a predicted tryptic fragment of the N-terminal hexahistidine-tagged portion of dCK (Table 1). The first two amino acids of this fragment, aspartate and proline, belong to the expression vector pEF6/His and are followed by Ala-2 of dCK; the initial methionine was absent because of the introduction of a BamHI restriction site in the dCK cDNA. The sequence of the tryptic peptide was confirmed by fragmentation, and the phosphorylated residue was identified as Thr-3. Peak III corresponded to a tryptic peptide of dCK containing two phosphates. Fragmentation of the tryptic peptide identified Ser-11 and Ser-15 as the phosphorylated residues. The tryptic peptide present in peak III was detected with four different monoisotopic masses (Table 1) because of alkylation of Cys-9 by iodoacetamide, covalent modification of this residue by acrylamide, or the presence of one or two phosphates. In peak IV, no phosphopeptide was detected, possibly because of poor ionization or because of ion suppression by other peptides in this fraction. By contrast, a phosphopeptide was detected in peak V, and Ser-74 was identified as the phosphorylated residue (Table 1). This phosphopeptide was also present with different isotopic masses because of acrylamide modification of Cys-59 or its alkylation by iodoacetamide and oxidation of Met-73.

In summary, analysis of HPLC-purified peptides by nano-ESI-MS/MS indicated that recombinant dCK was phosphorylated in vivo on at least four residues, Thr-3, Ser-11, Ser-15, and Ser-74. The relative...
**TABLE 2**

Conservation of dCK phosphorylation sites in vertebrate species

The following dCK or homologous protein amino acid sequences were obtained from NCBI: human (accession number A38585); mouse (accession number NP_31858.1); frog (Xenopus laevis, accession number AAH84070.1); chicken (Gallus gallus, accession number NP_001006451.1); fish (Danio rerio, accession number AAH83277.1; Tetraodon nigroviridis, accession number CAP7102.1). The sequences were aligned by ClustalW (www.ebi.ac.uk/clustalw/). The numbering at the top of each block of sequences corresponds to the identified phosphorylation sites in human dCK. Conserved residues are in boldface; phosphorylated residues are in red; acidic residues are in green.

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**FIGURE 5.** Effect of mutation of Ser-74 on dCK activity. dCK activity was measured in lysates of HEK 293T 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-poly(His) antibody. The results are the means ± S.E. of three independent experiments in which duplicate or triplicate transfections were performed. Normalized dCK activities from HEK 293T cells transfected with vector encoding wild type dCK were set as 100% because of variation of overexpression between independent experiments. For significance relative to the wild type dCK, *** indicates p < 0.001.

**FIGURE 6.** Effect of mutation of Ser-74 on dCK inactivation induced by sorbitol. HEK 293T cells transfected with vector encoding wild type (WT) dCK or the indicated mutants were treated with or without 0.5 M sorbitol for 1 h before measurement of dCK activity. dCK activities were normalized. The results are the means ± S.E. obtained from three parallel transfections.

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labeling of the HPLC peaks was calculated in three separate experiments. Calculation of the percentage of total $^{32}$P incorporation into each peak indicated that the major in vivo phosphorylation site of dCK was Ser-74 (peak V, 44.6 ± 6.2%), followed by Thr-3 (peak II, 10.9 ± 5.7%), and Ser-11/Ser-15 (peak III, 9.9 ± 1.7%). Peaks I and IV represented 22.3 ± 8.9 and 10.3 ± 3.7% of total $^{32}$P incorporation, respectively. It is noteworthy that the sequence surrounding Ser-74 is well conserved between human and other species (>66% identity) with dCK sequences from mouse, frog, chicken, and two fish, whereas the phosphorylation site sequences located in the N-terminal region of dCK are less conserved (Table 2). The sequence surrounding Ser-74 contains a series of acidic residues upstream from the phosphorylation site (at the −8, −7, −5, and −4 positions), followed by a leucine, a threonine, a glutamine, and a lysine at the −3, −2, +1, and +2 positions, respectively.

**Role of Ser-74 Phosphorylation on dCK Activity in HEK 293T Cells**—To test whether phosphorylation of Ser-74, the most heavily phosphorylated site found in vivo, was important for dCK activity, HEK 293T cells were transfected with mutated His-tagged dCK. Ser-74 was replaced by Ala to abolish phosphorylation at this locus (S74A mutant) or by Glu to mimic its phosphorylation (S74E mutant). The S74E mutation did not significantly modify dCK activity (Fig. 5), whereas the S74A mutation markedly decreased activity (by 94.1 ± 1.7%), suggesting that phosphorylation of Ser-74 is a crucial factor for dCK activity. Values of $K_m$ for deoxycytidine were 0.15 and 0.17 μM for the S74A and the S74E dCK mutants, respectively. Although treatment with λ-protein phosphatase strongly reduced wild type dCK activity (see Fig. 1), similar treatment did not affect dCK activity of the S74E mutant (30.7 ± 5.4 nmol/min/mg of protein after a 60-min treatment versus 28.9 ± 4.9 nmol/min/mg of protein for the untreated enzyme, n = 3), suggesting that the other phosphorylation sites of dCK are not, or not directly, implicated in the control of its catalytic activity. To verify this hypothesis, we constructed dCK mutants in which Thr-3, Ser-11, and Ser-15 were replaced by Ala or Glu. None of these mutations significantly modified dCK activity (results not shown).

HEK 293T cells overexpressing either the S74A or S74E mutants were incubated for 1 h with 0.5 m sorbitol. As illustrated in Fig. 6, both mutations prevented the ability of sorbitol to reduce dCK activity, as observed for wild type dCK, suggesting that sorbitol might act by promoting the dephosphorylation of Ser-74. To verify this hypothesis, a polyclonal anti-Ser-74 phosphopeptide antibody was generated and used to probe lysates of HEK 293T cells overexpressing wild type dCK treated with or without sorbitol. Fig. 7A, upper panel, corresponds to the immunoblot for the phosphospecific antibody, and Fig. 7B, lower panel, corresponds to a parallel blot probed with anti-poly(His) antibody as a control for dCK protein expression. The immunoblot in Fig. 7A, left panel, shows that dCK was recognized by the phosphospecific antibody. As expected, the signal was strongly decreased in lysates pretreated with A-protein phosphatase, in parallel with a decrease in dCK activity (Fig. 7C). The immunoblot in Fig. 7A, right panel, shows that dCK phosphorylation in cells treated with sorbitol was markedly decreased, suggesting that Ser-74 dephosphorylation was induced by sorbitol and was involved in the decrease in dCK activity measured in the same cells (Fig. 7C). When extracts from sorbitol-treated cells were incubated with A-protein phosphatase, the phosphorylation was undetectable, and dCK activity was further decreased.

**Phosphorylation of Ser-74 in Human Leukemic Lymphocytes**—Several arguments point to regulation of dCK by reversible phosphorylation in leukemic cells (19, 22, 24). To know whether Ser-74 phosphorylation might play a role in the control of dCK activity in these cells, we investigated the effects of agents that we found previously to increase dCK activity without changing dCK expression in leukemic cells (22, 24). CCRF-CEM cells were thus incubated with the topoisomerase-II inhibitor genistein or UV light-irradiated under conditions reported to produce maximal activation of dCK. After incubation, CCRF-CEM cell
lysates were probed either with the anti-phospho-Ser-74 antibody (Fig. 8A) or with the anti-dCK antibody (Fig. 8B). We could detect phosphorylation of Ser-74 in basal conditions. Moreover, we observed that Ser-74 phosphorylation increased (Fig. 8A) under conditions that enhanced dCK activity. Densitometric quantification showed that the extent of Ser-74 phosphorylation increased (Fig. 8B) in the lysates of the transfected cells. Activities are the means ± S.E. from the three parallel transfections.

DISCUSSION

Our study provides the direct demonstration that dCK, a key enzyme in the salvage pathway and in the activation of many nucleoside analogues used in anti-cancer and antiviral therapy, is constitutively phosphorylated and regulated in vivo by phosphorylation/dephosphorylation.

A widely used method to demonstrate the in vivo phosphorylation of a particular protein is to incubate intact cells with [32P]orthophosphate and to examine whether this protein is labeled. Because dCK phosphorylation could not be detected in leukemic cells incubated with [32P]orthophosphate because of low levels of expression, we overexpressed dCK in HEK 293T cells. The ability of A-protein phosphatase to dephosphorylate dCK activity in lysates of HEK 293T cells (Fig. 1) suggested that the recombinant dCK might be constitutively phosphorylated and that HEK 293T cells overexpressing dCK looked as a good model to study dCK phosphorylation. This approach allowed labeling dCK with [32P]orthophosphate, and after purification of dCK, tryptic digestion, and mass spectrometry analysis, identification of four phosphorylation sites on the protein, namely Thr-3, Ser-11, Ser-15, and Ser-74. Using the NetPhos 2.0 computer program (32), probability scores for phosphorylation of Ser-15 and Ser-74 were 0.993 and 0.982, respectively, 0.698 for Ser-11, and below the limit for potential phosphorylation of Thr-3 (0.404). Other phosphorylation sites probably exist as suggested by the labeling of peaks I and IV in the HPLC profile (Fig. 4), in which the phosphorylated residue was not identified.

The three-dimensional structure of human dCK was recently solved by Sabini et al. (33). The enzyme is homodimeric with a fold similar to that described for the Drosophila melanogaster deoxynucleoside kinase. Each monomer consists of 10 α-helices surrounding a five-stranded parallel β-sheet. The N-terminal extremity of dCK (residues 1–19) that contains three of the four phosphorylation sites identified is flexible, and its structure could not be solved. However, this region is predicted to lie outside the protein core. Ser-74 is located in a 15-residue mobile insert. Thus, the four phosphorylation sites we identified are located in flexible loops at the surface of the protein and would be accessible to protein kinases.

Ser-74 was identified as the major phosphorylated residue in 32P-labeled HPLC fractions. Its mutation to a nonphosphorylatable Ala residue strongly decreased the activity of dCK expressed in HEK 293T cells, whereas mutation of Ser-74 to Glu to mimic phosphorylation was without significant effect (Fig. 5). These results indicate that phosphorylation of Ser-74 is very important for dCK activity. That phosphorylation of Ser-74 is prevailing for the control of dCK activity is corroborated by our observation that activity of the S74E mutant was not decreased by λ-protein phosphatase treatment. Moreover, mutation of Thr-3, Ser-11, and Ser-15 to Ala did not acutely influence dCK activity, suggesting that phosphorylation of these residues is not essential for dCK activity. However, phosphorylation of these sites might be involved in other types of regulation, like enzyme stability or intracellular localization, which are currently under investigation.

Hyperosmotic stress induced by sorbitol was shown previously to decrease dCK activity (23) and was used as a tool to induce a change in dCK activity. As expected, we found that sorbitol decreased both dCK activity and 32P labeling of the protein, showing that activity of dCK can be correlated to its phosphorylation state. Moreover, mutation of Ser-74 to alanine or glutamate prevented the ability of sorbitol to reduce dCK

**FIGURE 7. Effect of sorbitol on Ser-74 phosphorylation in HEK 293T cells overexpressing dCK.** HEK 293T cells transfected with vector encoding wild type dCK were treated with or without 0.5 M sorbitol for 1 h before lysis. Lysates were then incubated with or without λ-protein phosphatase (λ-PP) for 1 h and subjected to SDS-PAGE followed by immunoblotting. A, dCK was detected with the specific anti-phospho-Ser-74 antibody or (B) with anti-poly(His) antibody as a control for dCK expression. Results from three parallel transfections are shown. The extent of Ser-74 phosphorylation (pSer74-dCK) expressed in arbitrary units, was added to the lysates. B, dCK activities in the lysates used for the immunoblots. Activities are the means ± S.E. from the three parallel transfections.

**FIGURE 8. dCK phosphorylation on Ser-74 in leukemic cells.** CCRF-CEM cells were incubated without (−) or with 100 μM genistein for 4 h or exposed to UV light (30 J/m²) and thereafter incubated for 30 min. Cell extracts (50 μg) were analyzed by SDS-PAGE followed by immunoblotting. dCK was detected with the specific anti-phospho-Ser-74 antibody (A) or with the anti-dCK antibody as a control for dCK expression (B). One representative experiment is shown.
activity, suggesting that dephosphorylation of Ser-74 occurred on treatment with sorbitol. This hypothesis was confirmed by use of an anti-phospho-Ser-74 antibody. Indeed, the signal detected with this specific antibody in untreated HEK 293T cells was almost abolished after incubation with sorbitol (Fig. 7). These results not only show that Ser-74 phosphorylation mirrors dCK activity but also provide an insight into the molecular mechanism by which sorbitol induces dCK inactivation. However, the signaling pathway by which sorbitol decreases Ser-74 phosphorylation remains to be elucidated.

Concerning the protein kinase(s) implicated in Ser-74 phosphorylation and the control of dCK activity, Prosite (www.expasy.org/tools/prosite/) (34) and Phosite (www.phosite.com) (35) searches indicate that Ser-74 could be a protein kinase C (PKC) site. Indeed, Wang and Kucera (36) found that dCK purified from leukemic blasts could be phosphorylated in vitro by PKCα. By contrast, human recombinant dCK, expressed in bacteria, was a very poor PKC substrate (37). Also, specific inhibitors or activators of PKC did not modify dCK activity in intact leukemic lymphocytes (24), suggesting that PKC does not play a specific role in the control of dCK activity in vivo. Further studies are thus required to identify the protein kinase(s) responsible for dCK phosphorylation. Regarding the dephosphorylation of dCK, protein phosphatase 2A might be directly or indirectly involved (24).

Recently, Keszler et al. (38) suggested that activation of dCK was accompanied by a conformational change. This hypothesis was based on the observation that extracts from CdA- or etoposide-treated cells were better recognized in native immunoblots by the antibody raised against dCK than extracts from control cells. Post-translational modification was proposed to induce a more open conformation, providing better accessibility for the antibody. Therefore, phosphorylation of Ser-74, which is located far from the active site, might induce long range conformational changes in dCK and promote an open conformation, as suggested by Keszler et al. (38).

Finally, an important aspect of this work is the finding that phosphorylation of Ser-74 was also detected in the leukemic CCRF-CEM cells and that Ser-74 phosphorylation was enhanced in these cells by agents (Fig. 8) known to increase dCK activity. These results strengthen a role for Ser-74 phosphorylation in the control of dCK activity, even in leukemic cells. These findings could be exploited for improving the activation of nucleoside analogues used in anti-cancer and antiviral chemotherapy or for the design of more active dCK mutants for suicide-gene therapy.

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