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Casein kinase 1δ activates human recombinant deoxycytidine kinase by Ser-74 phosphorylation, but is not involved in the *in vivo* regulation of its activity

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ABSTRACT

Deoxycytidine kinase (dCK) is a key enzyme in the salvage of deoxynucleosides and in the activation of several anticancer and antiviral nucleoside analogues. We recently showed that dCK was activated *in vivo* by phosphorylation of Ser-74. However, the protein kinase responsible was not identified. Ser-74 is located downstream a Glu-rich region, presenting similarity with the consensus phosphorylation motif of casein kinase 1 (CKI), and particularly of CKI δ. We showed that recombinant CKI δ phosphorylated several residues of bacterially overexpressed dCK: Ser-74, but also Ser-11, Ser-15, and Thr-72. Phosphorylation of dCK by CKI δ correlated with increased activity reaching at least 4-fold. Site-directed mutagenesis demonstrated that only Ser-74 phosphorylation was involved in dCK activation by CKI δ, strengthening the key role of this residue in the control of dCK activity. However, neither CKI δ inhibitors nor CKI δ siRNA-mediated knock-down modified Ser-74 phosphorylation or dCK activity in cultured cells. Moreover, these approaches did not prevent dCK activation induced by treatments enhancing Ser-74 phosphorylation. Taken together, the data preclude a role of CKI δ in the regulation of dCK activity *in vivo*. Nevertheless, phosphorylation of dCK by CKI δ could be a useful tool for elucidating the influence of Ser-74 phosphorylation on the structure–activity relationships in the enzyme.

Introduction

Human deoxycytidine kinase (dCK[4]; EC 2.7.1.74) catalyzes the phosphorylation of 2′-deoxycytidine (dCyd), 2′-deoxyadenosine and 2′-deoxyguanosine to their corresponding monophosphate forms, using ATP or UTP as phosphoryl donors. This reaction is the first and rate-limiting step of the deoxyribonucleoside salvage pathway, which provides deoxynucleoside triphosphates for DNA replication and repair as an alternative to *de novo* nucleotide synthesis [1]. In addition to recycling natural deoxynucleosides, dCK plays a key role in the activation of a number of anticancer and antiviral nucleoside analogues, including fludarabine, cladribine and cytarabine used in hematological malignancies, gemcitabine active against solid malignant tumors [2,3], and the anti-HIV compounds zalcitabine and lamivudine [4]. All these nucleoside analogues are prodrugs that need to be converted into triphosphate derivatives to exert their pharmacological action. As demonstrated by a positive correlation between dCK activity and nucleoside analogue sensitivity, this enzyme plays a critical role in their therapeutic efficacy [5].

The possibility of post-translational regulation of dCK activity was alluded to at the end of the 1990s, when it was found that several genotoxic drugs including the DNA polymerase α inhibitor aphidicolin, the topoisomerase II inhibitor etoposide, and certain nucleoside analogues, such as cladribine and cytarabine, increased dCK activity without changing its protein level [6–8]. Similar results were observed in response to UVC- and γ-irradiation, two treatments that induce DNA repair [9,10]. The presumption of a reversible phosphorylation as a mechanism for the regulation of

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[4] *Abbreviations used:* dCK, deoxycytidine kinase; CKI, casein kinase I; HEK, human embryonic kidney; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; WT, wild-type; OA, okadaic acid; APC; aphidicolin; dCyd, deoxycytidine; HIV, human immunodeficiency virus; UV, ultraviolet; CLL, chronic lymphocytic leukemia; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LB, Luria–Bertani; DTT, dithiothreitol; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; siRNA, silencing RNA; PKC, protein kinase C; ATM, ataxia telangiectasia mutated.

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dCK activity was strengthened by our observation that several protein kinase and phosphatase inhibitors could modulate dCK activity in several types of leukemic cells [11]. Also, we demonstrated that dCK overexpressed in HEK293T cells exists as a phosphoprotein that contains at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue. Site-directed mutagenesis experiments indicated that Ser-74 phosphorylation activated dCK, whereas phosphorylation of the other sites did not [12,13]. Moreover, use of a specific anti-phospho-Ser-74 antibody showed a direct correlation between the phosphorylation state of Ser-74 and dCK activity in human leukemic lymphocytes, including B-cell chronic lymphocytic leukemia (B-CLL) cells [14]. Although activation of dCK by Ser-74 phosphorylation is now well established, the signaling pathway involved in this process is not yet known. The first step toward deciphering this pathway would be to identify the protein kinase responsible for Ser-74 phosphorylation. This protein kinase should be constitutively active, since dCK is phosphorylated under basal conditions in various cultured cells [12,14]. By examining the sequence surrounding Ser-74 in dCK, we noticed that it was localized downstream a Glu-rich region presenting similarities with the consensus sequence of constitutively active casein kinase I (CKI) family, and particularly of CKI δ [15].

CKI isoforms are widely expressed and highly conserved Ser/Thr protein kinases. In mammals, at least seven distinct isoforms (α, β, γ1, γ2, γ3, δ and ε) have been identified, having a similar structural architecture with a short N-terminal region, a highly conserved catalytic domain of ~300 amino acids, and a highly variable C-terminal domain [16]. These kinases are implicated in a variety of cellular functions, including vesicle trafficking, DNA repair, cell division and progression, circadian rhythms and apoptosis [17]. Interestingly, CKI δ, like dCK, has been shown to display high activity in lymphocytes [18,19] and to play an important role in the DNA damage response [9,20,21]. Moreover, CKI δ protein levels were found to be p53-dependently elevated upon etoposide treatment [20] that also induces increase of Ser-74 phosphorylation and dCK activity in leukemic cells [7,14]. All these data made CKI δ a candidate enzyme for catalyzing Ser-74 dCK phosphorylation. Here, we investigated whether CKI δ could activate dCK *in vitro* and modulate dCK activity *in vivo*.

Experimental

Materials

Sequencing-grade trypsin was purchased from Promega. HPLC-grade solvents and acids were from Acros Organics. Primers for cloning and site-directed mutagenesis were from Eurogentec. Reverse transcriptase, T4 polynucleotide kinase, Tag polymerase, *Pfu* DNA polymerase, restriction enzymes and PageBlue™ Protein Staining Solution were from Fermentas. [5-^{3}H]-dCyd was from Moravek and [γ-^{32}P]-ATP from Perkin Elmer. Purified recombinant CKI δ expressed in bacteria was obtained from New England Biolabs (truncated at residue 317 to prevent autophosphorylation of its C-terminal domain and inhibition of casein kinase activity *in vitro* [22]). CKI-7 (*N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide) and α-casein were purchased from Sigma. IC-261 and okadaic acid (OA) were from Calbiochem. Anti-phospho-Ser-74 antibody was generated as described previously [12]. Anti-poly(His) monoclonal antibody was from GE Healthcare. Anti-CKI δ antibody and anti-CKI δ antibody coupled to agarose beads were from Santa Cruz Biotechnology. Anti-rabbit and anti-mouse IgG conjugated to peroxidase were from Sigma. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Pierce. Fluorescent secondary antibodies IRDye®800 or IRDye®680 and Odyssey blocking buffer were from LI-COR Biosciences. Other chemicals, materials and reagents were from Sigma, Calbiochem or Bio-Rad Laboratories.

Cell culture, transfection and treatment

B-CLL cells, isolated as previously reported [14], CCRF-CEM cells and HEK293T cells were cultured as described [12]. To generate HEK293T cells stably expressing human dCK, 4 μg of the eukaryotic pEF6/His vector containing the cDNA of human dCK was linearized with AatII and transfected in HEK293T cells using the JetPEI procedure (PolyPlus Transfection) [12]. Forty-eight hours after transfection, cells were cultured in selective medium containing 10 μg/ml blasticidin.

Plasmid construction and site-directed mutagenesis

The cDNA of human dCK was amplified by PCR with *Pfu* DNA polymerase using a 5′-primer containing the putative ATG codon in a NdeI site and a 3′-primer containing the putative stop codon flanked by a BamHI site. A ~800 bp product was obtained, which was subcloned in pBlueScript II SK (+) vector, checked by DNA sequencing and used for site-directed mutagenesis. Wild-type construct was used as a template (50–100 ng) to create single mutations by PCR (S74A or S74E) using the QuickChange protocol (Stratagene). 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. NdeI–BamHI fragments containing wild-type or mutated dCK were partially digested with NdeI and BamHI and ligated in pET-15b vector (Novagen) providing a His_6 tag at the N-terminus.

dCK expression and purification

The pET-15b vectors with the dCK constructs were used to transform *Escherichia coli* BL21 (DE3). The resulting bacteria were grown in Luria–Bertani (LB) medium containing 0.1 g/l ampicillin. The culture was grown at 37 °C until the A_{600} reached 0.5–0.6. It was then cooled on ice for 20 min and the inducer IPTG was added to a final concentration of 0.4 mM. After overnight incubation at 37 °C, bacteria were collected by centrifugation, resuspended in buffer A (50 mM HEPES, pH 7.5, 0.1% (w/v) β-mercaptoethanol, 20% (w/v) glycerol, 1 mg/ml lysozyme, 1 mM ρ-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml antipain) and submitted to three cycles of freezing and thawing. The bacterial extract was incubated on ice for 1 h with 0.1 mg/ml of DNaseI in the presence of 10 mM $MgSO_4$, and centrifugated for 30 min at 12,000*g*. The resulting supernatant (10 ml) was incubated with 1 ml of Talon (Co^{++}) affinity resin (Clonetech) for 1 h at 4 °C. After three washings with buffer B (50 mM HEPES, pH 7.5, 0.1% (w/v) β-mercaptoethanol, 1 mM ρ-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml antipain and 300 mM NaCl) followed by two washings with buffer B supplemented with 10 mM imidazole, recombinant dCK was eluted with buffer B containing 150 mM imidazole. Fractions containing purified protein were pooled, desalted using NAP-5 columns (GE Healthcare) equilibrated with 50 mM Tris–HCl, pH 7.6, 2 mM DTT and 5 mM benzamidine, and concentrated by vivaspin ultrafiltration spin column (Sartorius). The purified proteins were stored at −80 °C in the presence of 20% (w/v) glycerol.

Phosphorylation of recombinant dCK by CKI δ

Purified dCK (6 μg of protein) was incubated at 30 °C with 8000 units of CKI δ in 120 μl of phosphorylation buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 200 μM [γ-^{32}P]ATP (~1000 cpm/pmol). At various times,

aliquots (20 μl) were removed and stopped by boiling in Laemmli SDS–PAGE buffer. Samples were subjected to SDS–PAGE electrophoresis and autoradiography to visualize dCK labeling. To determine phosphorylation stoichiometry, the same gels were stained with Coomassie blue, and radioactivity of dCK bands was measured by Cerenkov counting. One unit of CKI δ corresponds to the amount of enzyme that catalyzes the incorporation of 1 pmol/min of ^{32}P at 30 °C.

To analyze whether CKI δ phosphorylated Ser-74 and increased dCK activity, incubation was performed as described above, except that 200 μM unlabeled ATP and 12 μg of purified dCK were used in a total volume of 120 μl and bovine serum albumin (BSA) (1 mg/ml) was included in the assay. At various times, aliquots (10 μl) were withdrawn for immediate measurement of dCK activity, as described below, or stopped in Laemmli SDS–PAGE buffer for the analysis of Ser-74 phosphorylation by immunoblotting.

Identification of phosphorylated residues by tandem mass spectrometry

For the identification of *in vitro* phosphorylated sites, purified dCK (10 μg) was incubated for 3 h with 8000 units of CKI δ and [γ-^{32}P]ATP as described above in a final volume of 30 μl. After the addition of 10 mM DTT and 100 mM iodoacetamide, the reaction was stopped by adding 10% trichloroacetic acid (v/v). After 30 min on ice, precipitated proteins were collected by centrifugation, washed once with 200 μl of ice-cold acetone and resuspended in 25 μl of 50 mM NH_4HCO_3 for overnight digestion at 30 °C with 1 μg of sequencing-grade trypsin. Peptides were separated by reverse-phase narrow-bore HPLC on a Vydac C18 column (2.1 mm × 25 cm) at a flow rate of 200 μl/min [23]. Radioactivity of each fraction was measured by Cerenkov counting. Radioactive peaks were concentrated to ~10 μl, 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), as described [12]. Fractions 19 + 20 were analyzed by MALDI TOF/TOF. One μl of a solution containing 5 mg/ml alpha-cyano-4-hydroxycinnamic acid (alpha-cyano MALDI matrix) in 50% (v/v) acetonitrile and 0.1% (v/v) TFA was added to 1 μl of peptide solution. One μl of this solution was spotted onto an Applied Biosystems MALDI plate Opti-TOF™ 384 Well Insert (Foster City, USA). MS and MS/MS spectra were acquired using an Applied Biosystems 4800 MALDI-TOF/TOF™ Analyzer spectrometer using a 200 Hz solid state laser operating at a wavelength of 355 nm. MS spectra were obtained using a laser intensity of 3000 and 2000 laser shots per spot in a range of m/z between 800 and 4000. MS/MS spectra were obtained by selecting 15 most intense precursors ions per spot and using a laser intensity of 3500 and 2000 laser shots per precursor. The automatically selected precursors were fragmented using a collision energy of 1 kV with collision gas air at a pressure of about 1×10^6 Torr. Data were collected with the Applied Biosystems 4000 Series Explorer™ software.

dCK assay

HEK293T, B-CLL and of CCRF-CEM cell extracts were prepared as reported previously [12,14]. The protein content of samples was measured by the method of Bradford [24] with BSA as a standard. dCK was assayed on 4–40 μg of cell extract or 0.15–1 μg of purified dCK. Except for kinetic studies, dCK activity was assayed in the presence of 10 μM dCyd and 5 mM ATP as described in [12]. The K_m for dCyd was determined with dCyd concentrations ranging from 0.02 to 2 μM. The Michaelis–Menten constants were calculated using non-linear regression with the GraphPad Prism 4.0 sofware.

Immunoblot analysis

Purified dCK (1 μg) or cell lysates (20–30 μg of protein) were subjected to SDS–PAGE electrophoresis in gels containing 12% (w/v) polyacrylamide and transferred to Hybond C-extra membranes (GE Healthcare) or low fluorescence PVDF immobilon-FL membranes (Millipore) depending on chemiluminescent (for CKI δ, GAPDH and β-actin) or fluorescent (for phospho-Ser-74, poly(-His)-dCK and dCK) detection. After transfer, Hybond C-extra membranes were blocked in PBS containing 5% (w/v) fat-free milk powder and then probed overnight at 4 °C with either anti-CKI δ antibody (1/1000), anti-GAPDH antibody (1/1000) or anti-β-actin antibody (1/10,000) in PBS-T (Tween 0.1%, w/v) with 5% (w/v) fat-free milk powder. After extensive washing in PBS-T, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase (1/10,000). After further extensive washings in PBS-T, the blots were developed using (advanced) enhanced chemiluminescence (GE Healthcare). Quantification of CKI δ expression was carried out using the ImageJ 1.42q software (http://rsb.info.nih.gov/ij). PDVF membranes were blocked in Odyssey blocking buffer. After washing in PBS-T (Tween 0.1%) and incubation with anti-phospho-Ser-74 antibody (1/1000), anti-poly(His) antibody (1/4000) or anti-dCK antibody (1/1000), the membranes were incubated for 1 h at room temperature with the appropriate fluorescent secondary antibody IRDye®800 or IRDye®680 (LI-COR Biosciences) diluted (1/10,000) 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 dCK expression or phosphorylation [13].

RNA interference

HEK293T cells stably expressing dCK were plated in 6 cm-dishes (0.5×10^6cells/dish) and transfected on the following day with the siRNA transfection reagent INTERFERin™ (PolyPlus Transfection) using 5 nM control siRNA, 5 nM GAPDH siRNA or 5 nM CKI δ siRNA (synthesized by Dharmacon) and 10 μl INTERFERin™, according to the manufacturer's instructions. After 48 h, the cells were washed with cold PBS and resuspended in lysis buffer containing 50 mM HEPES, pH 7.5, 50 mM NaF, 1 mM K_2HPO_4, 0.1% (w/v) β-mercaptoethanol, 5 mM β-glycerophosphate, 5 mM $Na_4P_2O_7$, 1% (w/v) Triton, protease inhibitors (1 mM *p*-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μg/ml leupeptin and antipain), 1 mM sodium orthovanadate and 20% glycerol (w/v). Cells were lysed by one cycle of freeze–thawing. After centrifugation, supernatants were boiled in Laemmli SDS–PAGE buffer and subjected to electrophoresis, or used for CKI δ assay, as described below.

CKI δ assay

For each CKI δ assay, a HEK293T cell lysate (500 μg of protein) was incubated overnight at 4 °C with 40 μl of agarose beads conjugated to anti-CKI δ antibody (sc-6474Ac). The beads were washed twice with 1 ml of lysis buffer without glycerol and Triton and twice with 1 ml of kinase buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 2 mM DTT, 10 mM NaF, 1 mM β-glycerophosphate and 1 mM orthovanadate). Immunoprecipitates were incubated in 20 μl of kinase buffer containing 10 μg of α-casein and 50 μM [γ-^{32}P]ATP (~1000 cpm/pmol). After 30 min at 30 °C, the reaction was terminated by addition of SDS sample buffer. ^{32}P-labeled α-casein was resolved by 12% SDS–PAGE and detected with Coomassie blue. The bands corresponding to α-casein were cut from the gel and subjected to counting by Cerenkov radiation.

Data analysis

The results of experiments repeated at least three times are given as means ± SEM. Significance was assessed by using the paired Student's *t* test.

Results

CKI δ phosphorylates and activates dCK

Inspection of the sequence surrounding Ser-74 of dCK showed a Glu-rich region upstream of the phospho-acceptor site, which fits the consensus recognition motif of CKI δ (Table 1). To determine whether dCK was a substrate for CKI δ, we tested commercially available CKI δ on human recombinant dCK expressed in *E. coli*. As illustrated in Fig. 1A, dCK was phosphorylated in a time-dependent manner on incubation with CKI δ and [γ-^{32}P]ATP. However, phosphorylation of dCK by CKI δ was less than with α-casein with 0.20 ± 0.03 mol of phosphate (n = 8) being incorporated per mol of dCK subunit after 1 h of incubation at 30 °C compared with 1.85 ± 0.41 mol of phosphate (n = 3) per mol of α-casein under the same conditions (not shown).

To verify whether CKI δ actually phosphorylated Ser-74, recombinant dCK was analyzed by Western blot with the specific anti-phospho-Ser-74 antibody. As illustrated in Fig. 1B, dCK was indeed phosphorylated with time on Ser-74 by CKI δ. In parallel, we observed that dCK activity progressively increased to attain a ∼ 4-fold activation after 4 h. (Fig. 1C). Neither phosphorylation of dCK on Ser-74 nor dCK activation appeared to be complete at this time. Additional experiments confirmed that dCK activation persisted at the same rate at least up to 6 h (not shown). No change in dCK activity was observed in the absence of CKI δ (Fig. 1C). IC-261, a selective CKI δ inhibitor, was found to reduce ^{32}P incorporation as well as Ser-74 phosphorylation and dCK activation by more than 80% (not shown).

CKI δ phosphorylates dCK on several residues

As the phosphorylation of protein substrate by CKI is not strictly dependent on the consensus sequence, depending also on the tertiary structure of its substrate [25], and can occur on multiple residues [26], we wanted to verify whether Ser-74 of dCK was the only site phosphorylated by CKI δ. Therefore, recombinant dCK was incubated for 3 h with CKI δ in the presence of [γ-^{32}P]ATP, precipitated with trichloroacetic acid and digested with trypsin. Peptides were separated by reverse-phase HPLC and fractions were counted by Cerenkov radiation. As the mixture also contained CKI δ peptides, we performed in parallel an analysis of a sample containing BSA instead of recombinant dCK, to confirm the absence of autophosphorylation sites in truncated CKI δ. No peak was found in this condition (not shown), whereas four peaks were detected with dCK (Fig. S1), indicating that CKI δ phosphorylated more than one residue in recombinant dCK. All the peaks were screened for phosphopeptides by neutral loss of H_3PO_4 (98 Da) by nano-ESI-MS/MS, as previously described [12]. In peak I, no phosphopeptide was detected. Peak II corresponded to a peptide of dCK containing one or two phosphates, which explains the detection of two different monoisotopic masses for this peptide (Table 2). Fragmentation of the tryptic peptides identified Ser-11 and Ser-15 as the phosphorylated residues. No phosphopeptide could be detected in peak III by nano-ESI-MS/MS. However, using MALDI TOF/TOF, a peptide corresponding to residues located upstream of the initiator Met and containing the poly(His) tag preceded by one or two phosphorylated Ser residues, was identified in peak III. Therefore, this phosphopeptide did not belong to native dCK. As peak IV was relatively broad, fractions 31–32 and 33–34 were analyzed separately. In fractions 31–32, a phosphopeptide was detected and Thr-72 was identified as the phosphorylated residue. Fractions 33–34 contained the same peptide, but phosphorylated on Ser-74. This phosphopeptide was also present with two different monoisotopic masses because of oxidation of one or two residues. Taken together, these results show that CKI δ phosphorylates recombinant dCK on at least four residues, Ser-11, Ser-15, Thr-72 and Ser-74. Peak IV that contains both phosphorylated Ser-74 and Thr-72 represented only 24.2 ± 2.5% of total ^{32}P incorporation, indicating that the phosphorylation of Ser-74 accounted for only a minor part of the total phosphorylation of dCK by CKI δ.

Phosphorylation of Ser-74 only plays a role in the activation of dCK by CKI δ

To determine whether phosphorylation of residues other than Ser-74 could contribute to the activation of dCK by CKI δ, we produced a mutated His-tagged dCK, in which Ser-74 was replaced by Ala (S74A mutant) to prevent phosphorylation at this locus. We also constructed the phosphomimetic S74E mutant, in which Glu mimics phosphorylation at Ser-74, to see whether phosphorylation at other sites might influence the activity of this activated form of dCK. The two mutated dCK proteins were produced in *E. coli* and purified by affinity chromatography, as for wild-type dCK. For S74A dCK, the specific activity was 38.4 ± 1.6 nmol/min/mg (n = 3), which was not drastically different from the activity of wild-type dCK (25.7 ± 2.8 nmol/min/mg, n = 6). By contrast, activity of the S74E mutant was 278.3 ± 17.6 nmol/min/mg (n = 3) and about 11-fold higher than the activity of wild-type dCK, as previously reported [27]. As illustrated in Fig. 2A, the activities of S74A and S74E dCK proteins were not significantly modified during the course of incubation with CKI δ (Fig. 2A). However, both mutants were clearly phosphorylated under these conditions (Fig. 2B). As expected from the absence of phosphorylatable Ser-74, labeling of S74A dCK was slightly lower (∼10% to 25%) than that of wild-type dCK, but more surprisingly, it was markedly lower for S74E dCK (∼50%) than for S74A dCK (Table S1). Both labeled mutated dCKs were subjected to analysis by nano-ESI-MS/MS, as described for wild-type dCK and the same phosphorylation sites, apart from Ser-74, were identified (not shown). These results indicate that phosphorylation of Ser-74 only plays a role in the activation of dCK by CKI δ and that phosphorylation of other sites does not inhibit the activity of S74E dCK.

Table 1
Comparison of sequence surrounding Ser-74 in dCK with the optimal sequence recognition motif of CKI δ determined by the peptide library approach [15]. Identical residues are shown in boldface. CKI δ is a negative charge-directed protein kinase, which needs a cluster of acidic amino acids located upstream the phosphorylated residue. dCK contains two Glu (E) residues at the −4 and −5 positions.

	Amino acid position										
	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5
Residues surrounding Ser-74 in dCK	**E**	**E**	L	**T**	M	**S**^{74}	Q	K	N	**G**	G
Optimal consensus sequence for CKI δ	**E**	F/**E**	D	**T**/A/G	G	**S**	I	I/I/F/Y/G	I/G/F	F/**G**	F/P/L

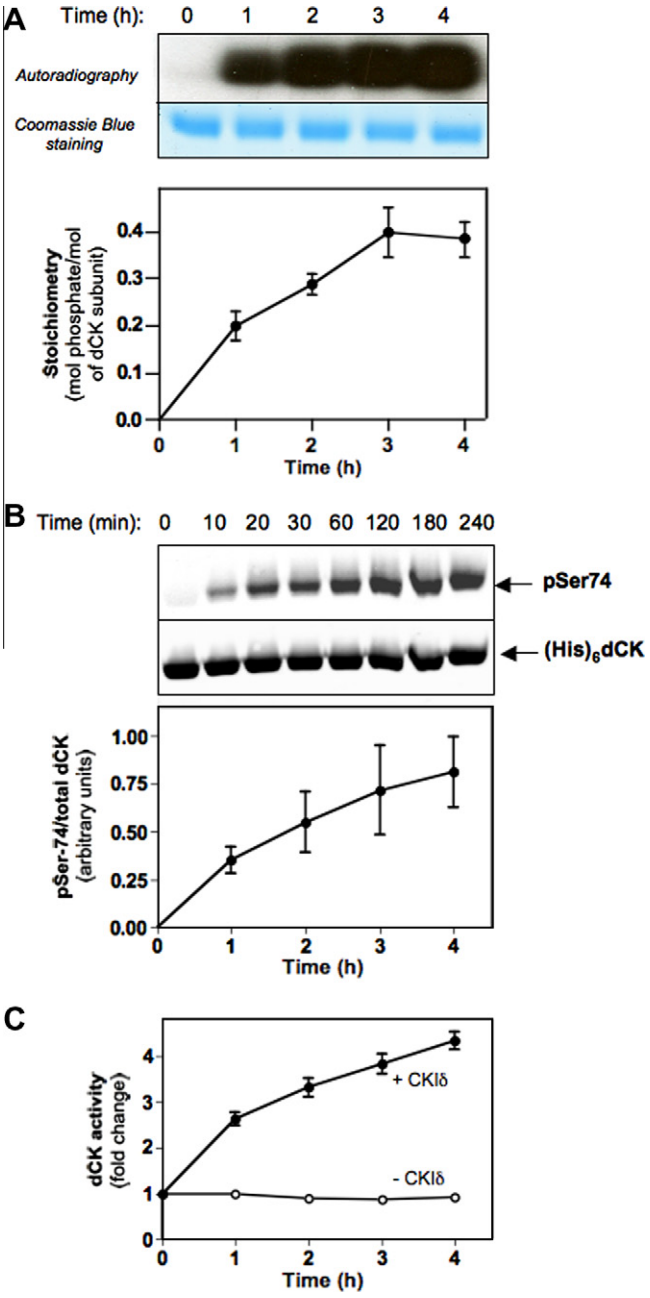

Fig. 1. Phosphorylation and activation of dCK by CKI δ *in vitro*. Purified human recombinant dCK was incubated in the presence of CKI δ and radioactive (A) or unlabeled (B and C) ATP, as described in the Methods. (A) At the indicated times, aliquots containing 1 μg of dCK were removed for SDS–PAGE, Coomassie blue staining and autoradiography (upper panels). The stoichiometry of phosphorylation was calculated after counting the radioactivity of dCK bands visualized by staining (lower panel). The results are means ± SEM of eight separate experiments. (B) At the indicated times, recombinant dCK phosphorylation by CKI δ was analyzed using anti-phospho-Ser-74 and anti-poly(His) antibodies by immunoblotting. One representative immunoblot and the pSer-74-dCK/total dCK ratios (means ± SEM of three independent experiments) are shown. (C) At various times of incubation in the presence or absence of CKI δ, dCK activity was measured with 10 μM dCyd as substrate. The results are the means ± SEM of 6 independent experiments. The specific activity of dCK before incubation was 25.7 ± 2.8 nmol/min/mg of protein ($n = 6$).

Phosphorylation of dCK by CKI δ changes its kinetic properties

The kinetic properties of dCK phosphorylated by CKI δ were studied and compared with those of unphosphorylated wild-type and S74E dCK. As shown in Table 3, phosphorylation of wild-type dCK by CKI δ for 3 h increased its K_{cat}, but which did not attain the K_{cat} value of S74E dCK. Moreover, phosphorylation of wild-type dCK by CKI δ markedly decreased its affinity for dCyd, the K_m value increasing about 4.5-fold. An increase in K_m, although lower, was also seen with the S74E mutant compared with the wild-type, confirming that phosphorylation of Ser-74 decreases the affinity of dCK for dCyd. As a result of parallel increases in K_{cat} and K_m, the catalytic efficiency (K_{cat}/K_m) of dCK after a 3 h-phosphorylation by CKI δ was not modified in comparison with unphosphorylated dCK, whereas it was increased about 3-fold for S74E dCK.

dCK activity is likely not regulated by CKI δ in cultured cells

To determine whether CKI δ phosphorylated dCK on Ser-74 and regulated its activity in intact cells, we first analyzed the effects of cell-permeable inhibitors of CKI δ. We incubated primary B-CLL lymphocytes with IC-261, a specific inhibitor of CKI δ/ε [28] or CKI-7, a general CKI inhibitor [29], at concentrations known to inhibit CKI δ in intact cells and we examined their influence on Ser-74 phosphorylation and on dCK activation induced by aphidicolin [14]. We observed (Fig. 3A) that Ser-74 phosphorylation, nearly undetectable in basal conditions, was clearly enhanced by aphidicolin, both in the absence and presence of IC-261 or CKI-7. Accordingly, these CKI inhibitors did not modify basal dCK activity or dCK activation induced by aphidicolin. Similar results were obtained in the leukemia cell lines CCRF-CEM and EHEB (not shown). We also analyzed the effect of CKI inhibitors in HEK293T cells, in which dCK is highly phosphorylated on Ser-74 after transient transfection [12]. Our strategy was to investigate whether CKI inhibition could prevent reactivation of dCK following its inactivation. To inactivate dCK, HEK293T cells were treated for 30 min with 0.5 M sorbitol which, as previously reported [12], induced a decrease in Ser-74 phosphorylation and dCK activity (Fig. 3B). The cells were then washed to remove sorbitol and incubated again for 90 min in the presence or absence of 200 μM CKI-7. At the end of this incubation period, Ser-74 phosphorylation had returned to its initial value in the absence, but also in the presence of CKI-7 (Fig. 3B), indicating that CKI is not responsible for the constitutive phosphorylation of dCK on Ser-74 in HEK293T cells.

A second approach to evaluate the contribution of CKI δ to Ser-74 phosphorylation in intact cells was to down-regulate the kinase by siRNA. As co-transfection of plasmids encoding dCK and CKI δ siRNA was unsuccessful in our hands, down-regulation of CKI δ was performed in HEK293T cells that stably expressed dCK. Unfortunately, these cells contained a much lower (at least 100-fold) level of dCK than HEK293T cells that transiently overexpressed dCK, so that Ser-74 phosphorylation was barely detectable in this model (Fig. 4A). Nevertheless, the level of Ser-74 phosphorylation could be increased and became detectable in these cells after incubation with okadaic acid (OA), a protein phosphatase 2A inhibitor (Fig. 4A). Therefore, we analyzed the consequences of CKI δ down-regulation in the presence of OA. Efficacy of siRNA treatment was assessed by analyzing the level of CKI δ protein by immunoblotting 48 h after transfection. Compared with control siRNA or GAPDH siRNA (that reduced GAPDH levels by about 50%), CKI δ siRNA decreased CKI δ levels by about 60% (Fig. 4B and C). A marked decrease in CKI δ electrophoretic mobility in the presence of OA was observed, which could be explained by an increase in autophosphorylation on its carboxy-terminal domain. Indeed, CKI δ, like the closely related CKIε, autophosphorylates on C-terminal Ser and Thr residues, creating an autoinhibitory domain for its kinase activity [22,30]. In fact, OA induced a marked decrease in CKI δ activity in HEK293T cells (Fig. 4D). CKI δ siRNA reduced CKI δ activity by about 60% in parallel with the decrease in its protein

Table 2
Sequences of dCK phosphopeptides obtained by mass spectrometry after phosphorylation by CKI δ. HPLC peaks (Fig. S1) were analyzed by nano-ESI-MS/MS or *MALDI TOF/TOF. Phosphopeptides were identified by loss of 98 Da upon collision-induced dissociation, and the phosphorylated residue was further identified by fragmentation. c, iodoacetamide (carboxamidomethyl)-modified cysteine; p, phosphorylated residue; M_{ox}, oxidized Met; ND, not determined.

Peptide	Fractions	Sequence	Measured average masses [M+H⁺]	Theoretical monoisotopic masses [M+H⁺]
I	10	ND		
II	13 + 14	^{8}ScPpSFSApSSEGTR^{20}	1532.4 (avg)	1532.5
	15	^{8}ScPpSFSASSEGTR^{20}	1452.5	1452.5
III	*19 + 20	$^{-19}$GpSSHHHHHHSSGLVPR^{-4}	1848.86	1848.81
		$^{-19}$GpSpSHHHHHHSSGLVPR^{-4}	1928.83	1928.78
IV	31 + 32	^{58}WcNVQSTQDEFEELpTMpSQK^{7}	2520.1 (avg)	2519.9
	33 + 34	^{58}WcNVQSTQDEFEELTMoxpSQK^{76}	2455.8	2455.9
		^{58}WoxcNVQSTQDEFEELTMoxpSQK^{76}	2459.8	2460.0

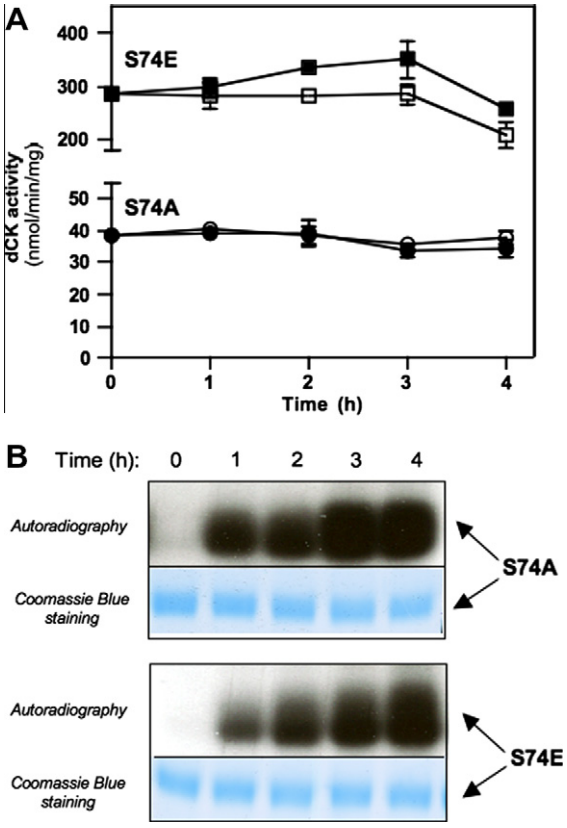

Fig. 2. Effect of mutation of Ser-74 on dCK activity after phosphorylation by CKI δ. (A) Recombinant S74A and S74E dCK proteins were incubated with unlabeled ATP in the presence (●, ■) or absence (○, □) of CKI δ. dCK activity was measured at the indicated times. The results are the means ± SEM of 3 independent experiments. (B) The recombinant S74A and S74E proteins were incubated in the presence of CKI δ and [γ-^{32}P]ATP. At the indicated times, aliquots corresponding to 1 μg of dCK were taken for SDS–PAGE and autoradiography. Representative data are shown.

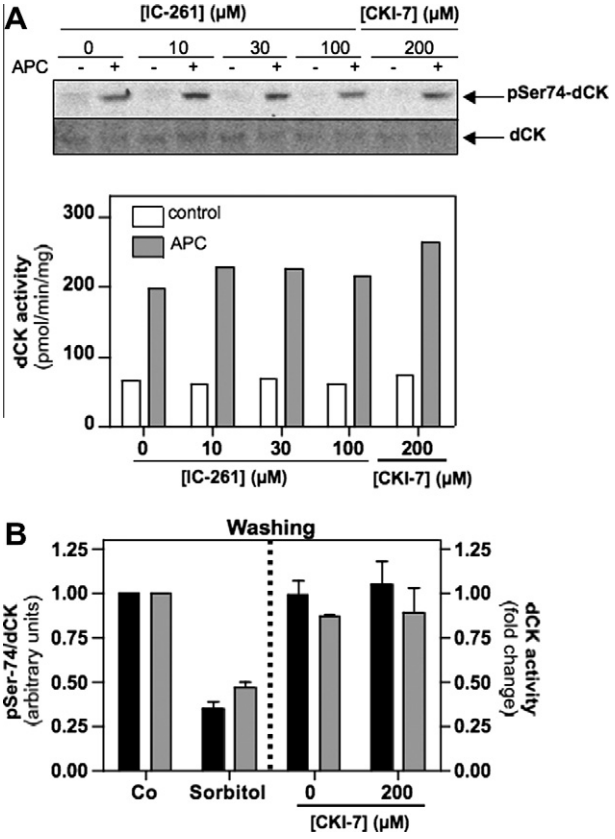

Fig. 3. Effect of CKI inhibitors on Ser-74 phosphorylation and dCK activity *in vivo*. (A) Freshly isolated B-CLL cells were pre-treated, or not, with increasing concentrations of IC-261 or with 200 μM CKI-7 for 30 min, and then incubated for 2 h in the presence or absence of 10 μM aphidicolin (APC). Cell lysates were analyzed for dCK phosphorylation and expression by immunoblotting with the anti-phospho-Ser-74 and anti-dCK antibodies (upper panel) and for dCK activity (lower panel). A representative experiment is shown. (B) HEK293T cells were transiently transfected with the PEF_6/His vector containing wild-type dCK. Forty hours after transfection, HEK293T cells were incubated for 30 min in the presence or absence of 0.5 M sorbitol to decrease Ser-74 phosphorylation and dCK activity. Then, cells were washed to remove sorbitol and incubated for 90 min with or without 200 μM CKI-7. Before washing and after incubation with or without CKI-7, cells were lysed and analyzed for dCK Ser-74 phosphorylation by immunoblotting (black bars) and for dCK activity (gray bars). The results are the means ± SEM of 3 separate experiments.

Table 3
Kinetic parameters of wild-type dCK, dCK phosphorylated by CKI δ and S74E dCK. Wild-type dCK was incubated for 3 h at 30 °c with or without CKI δ and ATP. Kinetic measurements were performed with 5 mM ATP and increasing concentrations of dCyd from 0.02 to 2 μM. For comparison, the kinetic parameters of S74E dCK, also incubated for 3 h at 30 °c, were determined. The results are the means ± SEM of three or five separate experiments, as indicated.

	Wild-type (n = 5)	Wild-type + CKI δ (n = 3)	S74E (n = 3)
K_{cat} (s^{-1})	0.016 ± 0.001	0.078 ± 0.010	0.153 ± 0.024
K_m (nM)	59 ± 3	268 ± 31	157 ± 27
K_{cat}/K_m (M 10^{-9} s^{-1})	281 ± 12	289 ± 13	983 ± 40

level. A combination of both siRNA CKI δ and OA treatment led to more than 90% inhibition of CKI δ activity (Fig. 4D). Despite this acute decrease in activity, neither phosphorylation of Ser-74 nor dCK activity were modified (Fig. 4E), indicating that CKI δ was likely not responsible for dCK Ser-74 phosphorylation in intact cells.

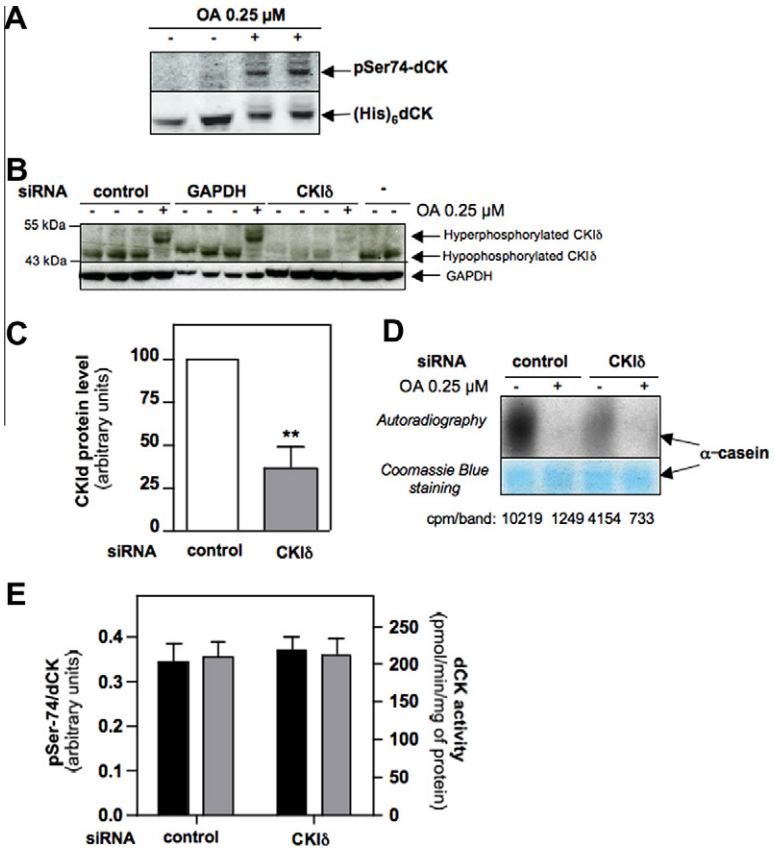

Fig. 4. Effect of CKI δ down-regulation by siRNA on Ser-74 phosphorylation and dCK activity in HEK293T cells stably expressing dCK. (A) Cells were incubated in the presence or absence of 0.25 μM okadaic acid (OA) for 4 h. Cell lysates were probed for Ser-74 dCK phosphorylation and expression with anti-phospho-Ser-74 and anti-poly(His) antibodies by immunoblotting. A representative immunoblot is shown. (B) Cells were transfected with 5 nM control siRNA, 5 nM GAPDH siRNA or 5 nM CKI δ siRNA for 48 h in quadruplicate. In one, 0.25 μM OA was added 4 h before the end of incubation. CKI δ and GAPDH expression were analyzed by immunoblotting after loading 30 μg of protein, as illustrated in a representative immunoblot. (C) CKI δ and GAPDH expression was quantified by ImageJ software. The results shown are the means ± SEM of three independent experiments. ** Significant relative to control siRNA: $P < 0.01$. (D) HEK293T cells were transfected with control siRNA or CKI δ siRNA for 48 h and 0.25 μM OA was added 4 h before the end of incubation. After cell lysis and immunoprecipitation, CKI δ activity was measured as explained in the Methods. A representative experiment is shown. (E) HEK293T cells were transfected with 5 nM control siRNA or 5 nM CKI δ siRNA for 48 h and 0.25 μM OA was added 4 h before the end of incubation. Lysates were probed for Ser-74 dCK phosphorylation (black bars) and activity (gray bars). The results are the means ± SEM of three independent experiments.

Discussion

Regulation of dCK activity by Ser-74 phosphorylation was discovered by our group four years ago [12]. However, a protein kinase able to phosphorylate this residue has not been identified since. Similarities between the sequence surrounding Ser-74 in dCK and the recognition motif of CKI δ led us to investigate the potential role of this kinase in the regulation of dCK activity. Our study shows that dCK can be phosphorylated by CKI δ *in vitro* on Ser-74 and that phosphorylation correlated with dCK activation. However, CKI δ is unlikely to be the kinase responsible for Ser-74 phosphorylation and control of dCK activity in intact cells.

Initial studies characterizing the substrate specificity of CKI isoforms led to the identification of the consensus sequence pS/T-X-X-S/T, where pS/T refers to a phospho-Ser or a phospho-Thr, X refers to any amino acid and the underlined residues refer to the target site [31,32], indicating that phosphorylation of a Ser/Thr residue by CKI requires priming by another kinase. Nevertheless, further studies showed that CKI also phosphorylates a related unprimed site, which optimally contains a cluster of acidic amino acids N-terminal to the target S/T including an acidic residue at the n-3 position [33,34]. The sequence upstream of Ser-74 does not possess an acidic residue at n-3 position, but nevertheless contains two Glu at n-5 and n-4 positions in addition to a Thr at the n-2 position, which are all three found in the optimal consensus sequence of CKI δ (Table 1). We indeed demonstrated that CKI δ was able to phosphorylate dCK (Fig. 1A) and more precisely to phosphorylate Ser-74 (Fig. 1B), as confirmed by use of a specific anti-phospho-Ser-74 antibody. Moreover, as revealed by tryptic digestion and mass spectrometry, residues other than Ser-74 were phosphorylated by CKI δ, namely Ser-11, Ser-15 and Thr-72 (Fig. S1 and Table 2). The HPLC peak IV that contained Ser-74, but also Thr-72 (Fig. S1 and Table 2), represented 24.2 ± 2.5% ($n = 3$) of total ^{32}P incorporation, indicating that Ser-74 phosphorylation does not exceed ¼ of the overall stoichiometry. Although the sequences surrounding Ser-11 and Ser-15 do not fit the CKI consensus as well as that of Ser-74, they were phosphorylated by CKI δ presumably reflecting dependence on the tertiary structure of the substrate [25]. This emphasizes that the consensus sequences found in databases are not always strictly adhered to. It is noteworthy that phosphorylation of Ser-11 and Ser-15 in addition to Ser-74 of dCK has been detected *in vivo* [12]. dCK activity clearly increased in a time-dependent manner on incubation with CKI δ, reaching more than 4-fold activation after a 4 h-incubation (Fig. 1C). This corresponds to the maximal activation of dCK induced by DNA-damaging agents in leukemic cells [11,14]. As sites other than Ser-74 were phosphorylated by CKI δ, it was essential to investigate the role of the phosphorylation of these sites in the activation of dCK by CKI δ. Studies with S74A dCK indicated that dCK activation by CKI δ resulted only from Ser-74 phosphorylation, since no activation of S74A dCK was observed, despite phosphorylation at other sites (Fig. 2B). These results corroborate our previous finding that

phosphorylation of Ser-74 plays a crucial role in the control of dCK activity and confirm that phosphorylation of Ser-11 and Ser-15 does not influence dCK activity [13]. Also, phosphorylation of Thr-72, which had not been detected *in vivo*, seems not to influence dCK activity. When S74E dCK was phosphorylated by CKI δ, no major change in dCK activity was noted (Fig. 2A). However, a ~50% reduction in its labeling was observed (Table S1), suggesting that phosphorylation of dCK at Ser-74 by CKI δ could hamper phosphorylation at the other sites. Another possibility is that the three-dimensional conformation of S74E dCK is less favorable to phosphorylation by CKI δ than that of S74A.

Kinetic studies performed by McSorley et al. [27] had shown that replacement of Ser-74 by Glu in dCK increased both the K_m (~5-fold) and the K_{cat} (~11-fold) of dCK, measured with dCyd as substrate, resulting in a ~2-fold increase in catalytic efficiency (K_{cat}/K_m) [27]. We found that phosphorylation of dCK by CKI δ also increased the K_m for dCyd (4.5-fold), and the K_{cat} (4.9-fold). However, the catalytic efficiency of dCK phosphorylated at Ser-74 by CKI δ did not attain that of S74E dCK, so that the catalytic efficiency of dCK was not modified by phosphorylation. This discrepancy could be explained by the fact that dCK was not maximally phosphorylated by CKI δ after a 3 h-incubation or by the difference between a Glu and phosphorylated Ser residue.

Since dCK could be activated *in vitro* by CKI δ, we thought that dCK could also be activated by this kinase *in vivo*, which was tested by two different approaches. First, CKI-7, a general CKI inhibitor, and IC-261, considered as a more selective CKI δ inhibitor, were unable at effective concentrations [28,35–37] to decrease or to prevent Ser-74 dCK phosphorylation in various cultured cells (HEK293T cells transiently overexpressing dCK, B-CLL or CCRF-CEM cells) either under basal or dCK activating conditions (Fig. 3). Also, the two inhibitors did not influence basal dCK activity or dCK activation. Second, specific down-regulation of CKI δ by RNA interference did not decrease Ser-74 phosphorylation or dCK activity in HEK293T cells that stably expressed dCK (Fig. 4). However, CKI δ activity was reduced by more than 90% in our experimental model because of both a reduction in CKI δ protein level and treatment with OA. The latter was added to allow detection of Ser-74 dCK phosphorylation by immunoblotting, but also induced hyperphosphorylation of CKI δ and inhibition of its activity, as previously reported [22,30]. Taken together, the results indicate that CKI δ is not implicated in the control of dCK activity or its activation by DNA-damaging agents such as aphidicolin, most probably because Ser-74 of dCK is a poor substrate of this protein kinase. A role of another member of the CKI family is also unlikely since the general CKI inhibitor, CKI-7, did not affect Ser-74 phosphorylation or dCK activity. The protein kinase responsible for Ser-74 phosphorylation and for the control of dCK activity *in vivo* thus remains to be identified. Several years before identification of Ser-74 as a crucial phosphorylation site for the control of dCK activity, it was reported that dCK purified from leukemic blasts could be phosphorylated and activated by protein kinase C (PKC) α [38]. However, this was not the case when bacterially expressed human recombinant dCK was used as substrate of PKC [39]. Furthermore, specific activators or inhibitors of PKC did not modify dCK activity in intact leukemic cells [11], precluding a role of PKC in the control of dCK activity *in vivo*. As suggested by a proteomic study, Ser-74 could be a target of ATM (ataxia telangiectasia mutated) kinase [40], but this remains to be demonstrated. We are currently trying to identify the Ser-74-dCK kinase by fractionating cell extracts by chromatography techniques.

In conclusion, CKI δ can phosphorylate dCK *in vitro* at several sites. Among these, only phosphorylation at Ser-74 controls dCK activity. To date, CKI δ is the only known protein kinase that can phosphorylate dCK on Ser-74. Although, it is unlikely to be responsible for the control of dCK activity *in vivo*, it could be used as a tool to produce a phosphorylated and active form of dCK for the study of structure–activity relationships via phosphorylation on Ser-74.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.07.009.

References

[1] E.S. Arner, S. Eriksson, Pharmacol. Ther. 67 (1995) 155–186.
[2] C.M. Galmarini, J.R. Mackey, C. Dumontet, Lancet Oncol. 3 (2002) 415–424.
[3] E. Van Den Neste, S. Cardoen, F. Offner, F. Bontemps, Int. J. Oncol. 27 (2005) 1113–1124.
[4] E. De Clercq, H. Field, Br. J. Pharmacol. 147 (2006) 1–11.
[5] V. Verhoef, J. Sarup, A. Fridland, Cancer Res. 41 (1981) 4478–4483.
[6] M. Sasvari-Szekely, T. Spasokoukotskaja, M. Szoke, Z. Csapo, A. Turi, I. Szanto, S. Eriksson, M. Staub, Biochem. Pharmacol. 56 (1998) 1175–1179.
[7] T. Spasokoukotskaja, M. Sasvari-Szekely, G. Keszler, F. Albertioni, S. Eriksson, M. Staub, Eur. J. Cancer 35 (1999) 1862–1867.
[8] T. Spasokoukotskaja, M. Sasvari-Szekely, L. Hullan, F. Albertioni, S. Eriksson, M. Staub, Adv. Exp. Med. Biol. 431 (1998) 641–645.
[9] E. Van Den Neste, C. Smal, S. Cardoen, A. Delacauw, J. Frankard, A. Ferrant, G. Van den Berghe, F. Bontemps, Biochem. Pharmacol. 65 (2003) 573–580.
[10] Z. Csapo, G. Keszler, G. Safrany, T. Spasokoukotskaja, I. Talianidis, M. Staub, M. Sasvari-Szekely, Biochem. Pharmacol. 65 (2003) 2031–2039.
[11] C. Smal, S. Cardoen, L. Bertrand, A. Delacauw, A. Ferrant, G. Van den Berghe, F. Bontemps, Biochem. Pharmacol. 68 (2004) 95–103.
[12] C. Smal, D. Vertommen, L. Bertrand, S. Ntamashimikiro, M.H. Rider, E. Van Den Neste, F. Bontemps, J. Biol. Chem. 281 (2006) 4887–4893.
[13] C. Smal, S. Ntamashimikiro, A. Arts, E. Van Den Neste, F. Bontemps, Nucleosides Nucleotides Nucleic Acids 29 (2010) 404–407.
[14] C. Smal, E. Van Den Neste, M. Maerevoet, X. Poire, I. Theate, F. Bontemps, Cancer Lett. 253 (2007) 68–73.
[15] Z. Songyang, K.P. Lu, Y.T. Kwon, L.H. Tsai, O. Filhol, C. Cochet, D.A. Brickey, T.R. Soderling, C. Bartleson, D.J. Graves, A.J. DeMaggio, M.F. Hoekstra, J. Blenis, T. Hunter, L.C. Cantley, Mol. Cell. Biol. 16 (1996) 6486–6493.
[16] S.D. Gross, R.A. Anderson, Cell. Signal. 10 (1998) 699–711.
[17] U. Knippschild, A. Gocht, S. Wolff, N. Huber, J. Lohler, M. Stoter, Cell. Signal. 17 (2005) 675–689.
[18] T. Maritzen, J. Lohler, W. Deppert, U. Knippschild, Eur. J. Cell Biol. 82 (2003) 369–378.
[19] T. Spasokoukotskaja, E.S. Arner, O. Brosjo, P. Gunven, G. Juliusson, J. Liliemark, S. Eriksson, Eur. J. Cancer 31A (1995) 202–208.
[20] U. Knippschild, D.M. Milne, L.E. Campbell, A.J. DeMaggio, E. Christenson, M.F. Hoekstra, D.W. Meek, Oncogene 15 (1997) 1727–1736.
[21] O. Alsheich-Bartok, S. Haupt, I. Alkalay-Snir, S. Saito, E. Appella, Y. Haupt, Oncogene 27 (2008) 3653–3661.
[22] P.R. Graves, P.J. Roach, J. Biol. Chem. 270 (1995) 21689–21694.
[23] A. Woods, D. Vertommen, D. Neumann, R. Turk, J. Bayliss, U. Schlattner, T. Wallimann, D. Carling, M.H. Rider, J. Biol. Chem. 278 (2003) 28434–28442.
[24] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[25] A. Cegielska, I. Moarefi, E. Fanning, D.M. Virshup, J. Virol. 68 (1994) 269–275.
[26] R.B. Pearson, B.E. Kemp, Methods Enzymol. 200 (1991) 62–81.
[27] T. McSorley, S. Ort, S. Hazra, A. Lavie, M. Konrad, FEBS Lett. 582 (2008) 720–724.
[28] L. Behrend, D.M. Milne, M. Stoter, W. Deppert, L.E. Campbell, D.W. Meek, U. Knippschild, Oncogene 19 (2000) 5303–5313.
[29] T. Chijiwa, M. Hagiwara, H. Hidaka, J. Biol. Chem. 264 (1989) 4924–4927.
[30] A. Rivers, K.F. Gietzen, E. Vielhaber, D.M. Virshup, J. Biol. Chem. 273 (1998) 15980–15984.
[31] H. Flotow, P.R. Graves, A.Q. Wang, C.J. Fiol, R.W. Roeske, P.J. Roach, J. Biol. Chem. 265 (1990) 14264–14269.
[32] H. Flotow, P.J. Roach, J. Biol. Chem. 264 (1989) 9126–9128.
[33] H. Flotow, P.J. Roach, J. Biol. Chem. 266 (1991) 3724–3727.
[34] V. Pulgar, O. Marin, F. Meggio, C.C. Allende, J.E. Allende, L.A. Pinna, Eur. J. Biochem. 260 (1999) 520–526.

[35] M. Winter, D. Milne, S. Dias, R. Kulikov, U. Knippschild, C. Blattner, D. Meek, Biochemistry 43 (2004) 16356–16364.
[36] G. Li, H. Yin, J. Kuret, J. Biol. Chem. 279 (2004) 15938–15945.
[37] P.V. Agostino, S.A. Plano, D.A. Golombek, J. Genet. 87 (2008) 467–471.
[38] L.M. Wang, G.L. Kucera, Biochim. Biophys. Acta 1224 (1994) 161–167.
[39] T. Spasokoukotskaja, Z. Csapo, M. Sasvari-Szekely, S. Virga, I. Talianidis, S. Eriksson, M. Staub, Adv. Exp. Med. Biol. 486 (2000) 281–285.
[40] S. Matsuoka, B.A. Ballif, A. Smogorzewska, E.R. McDonald 3rd, K.E. Hurov, J. Luo, C.E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S.P. Gygi, S.J. Elledge, Science 316 (2007) 1160–1166.