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Activation of deoxycytidine kinase in lymphocytes is calcium dependent and involves a conformational change detectable by native immunostaining

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Abstract

Deoxycytidine kinase (dCK), the principal deoxynucleoside salvage enzyme, plays a seminal role in the bioactivation of a wide array of cytotoxic nucleoside analogues. Recently, activation of dCK has been considered as a protective cellular response to a number of DNA-damaging agents in lymphocytes. Regarding the molecular mechanism of the enzyme activation, a post-translational modification by protein phosphorylation has been suggested. Here we provide evidence that both the activation process and the maintenance of the activated state require free cytosolic calcium. BAPTA-AM, a cell-permeable calcium chelator selectively inhibited the activation of dCK in a time- and concentration-dependent manner while extracellular calcium depletion had no effect. On the other hand, elevation of cytoplasmic calcium levels by thapsigargin did not potentiate the enzyme, referring to the permissive function of calcium in the activation process. Denaturing Western blots of extracts from lymphocytes incubated with 2-chlorodeoxyadenosine, aphidicolin and/or BAPTA-AM clearly demonstrated that dCK protein levels were unchanged during these treatments. However, a striking correlation was found between enzyme activity and the intensity of dCK-specific signals in native Western blots. Extracts from CdA-treated cells were much better recognized by the antibody raised against the C-terminal peptide of dCK than the BAPTA-AM-treated samples. These results indicate that the calcium-dependent activation of dCK is accompanied by a conformational change that renders the C-terminal epitope more accessible to the antibody.

Keywords: Deoxycytidine kinase; 2-Chlorodeoxyadenosine (Cladribine); Calcium; BAPTA-AM; Conformational change; Native immunoblot

1. Introduction

The adequate maintenance of intracellular deoxyribonucleotide pools to supply the needs of DNA replication, repair and recombination is a central issue of nucleotide metabolism, accomplished by the tightly regulated pathways of the *de novo* and salvage biosynthesis. In resting cells, such as lymphocytes or neurons in the central nervous system, the salvage reactions dominate over the *de novo* synthesis. Deoxycytidine kinase (dCK; EC 2.7.1.74) plays a preponderant role in the salvage pathway due to its ability to provide all the four natural deoxyribonucleotides [1,2]. Recently, dCK has gained a special clinical interest since this enzyme catalyzes the rate-limiting step in the bioactivation of several nucleoside analogue prodrugs, widely used in anticancer treatments [3,4]. Numerous studies have set up a direct correlation between dCK activity levels and the efficacy of chemotherapy [5–7].

Previously we have observed that short-term treatments of normal and leukemic lymphocytes with deoxynucleoside analogues—such as 2-chloro-2′-deoxyadenosine (CdA)—result in profound upregulation of dCK activity

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Abbreviations: dCK, deoxycytidine kinase; TK, thymidine kinase; ^{3}HdT, 2′-deoxy-[5-methyl-^{3}H]thymidine; ^{3}HdC, 2′-deoxy-[5-^{3}H]cytidine; dC, 2′-deoxycytidine; dT, 2′-deoxythymidine; CdA, 2-chloro-2′-deoxyadenosine; DEAE, diethylaminoethyl; PVDF, polyvinylidene fluoride; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; DTT, 1,4-dithio-L-threitol; PKC, protein kinase C; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester).

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which is not accompanied by elevation in the protein levels [8,9]. Similar effects were evoked by a range of non-nucleoside agents including the DNA polymerase inhibitor aphidicolin, hydrogen peroxide, UV- and γ-irradiation, as well as by protein phosphatase inhibitors [10–12]. The fact that stimulated dCK enzyme activity could be completely abrogated by λ protein phosphatase treatment of the purified enzyme led us to propose that the activation process should involve a reversible phosphorylation step [10,12].

The biological relevance of the elevated dCK activity in cells exposed to genotoxic stress has been interpreted as a protective cellular response in terms of promoting DNA repair by enforced production of dNTP precursors [11,12]. On the other hand, prolonged treatments of cells with these DNA-damaging agents will eventually elicit apoptosis that provides the basis for their therapeutic effect. Programmed cell death is executed by activation of the p53-initiated signaling pathway and/or through a direct toxic effect on the mitochondria that is mediated by transient cytoplasmic Ca^{2+} surges [13,14]. Since nucleoside analogues initiate both the calcium currents and the activation of dCK, it seemed reasonable to explore if these events are connected, namely whether calcium signaling takes part in the potentiation of dCK activity.

In this paper we demonstrate that free cytoplasmic calcium is an indispensable factor for both the activation of dCK and maintaining its active state in cells. Furthermore, we provide an insight into the molecular mechanism of dCK activation based on native immunoblots. Our results suggest that dCK adopts a more open conformation upon activation, and establish a direct correlation between the conformational state and the activity of dCK.

2. Materials and methods

2.1. Chemicals

2′-Deoxy-[5-methyl-^{3}H]thymidine (^{3}HdT, 925 GBq/mmol, 37 MBq/mL), 2′-deoxy-[5-^{3}H]cytidine (^{3}HdC, 555 GBq/mmol, 37 MBq/mL), Hybond-P PVDF transfer membranes, Hybond ECL nitrocellulose membranes, anti-rabbit IgG and the Western blotting detection system were purchased from Amersham Life Sciences. Non-labeled nucleosides, phenylmethylsulphonyl fluoride, dithiothreitol, Nonidet P-40, glycerol, Ponceau S, BAPTA-AM, thapsigargin, Coomassie Brilliant Blue R 250 and EGTA were obtained from Sigma Chemical Co, protein assay reagents and the broad-range protein marker from Bio-Rad Laboratories and Eagle's minimal essential medium from the National Institute of Public Health. The rest of the chemicals were of analytical grade and produced by Reanal.

2.2. Cell cultures and treatments

Lymphocytes were freshly isolated from surgically removed palatine tonsils of 3- to 6-year-old children as previously described [2]. Short-term primary cultures (10^7 cells/mL) were maintained in Eagle's minimal essential medium at 37° and treated with the indicated drugs for 2 hr.

2.3. Preparation of cell extracts and assay of dCK and TK enzyme activities

Control and treated cell cultures containing 5×10^6 cells were pelleted by centrifugation at 950 *g* for 2 min at 4°, washed twice with 1× PBS and extracted in 100 μL lysis buffer composed of 50 mM Tris–HCl pH 7.6, 2 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 20% (v/v) glycerol and 0.5% Nonidet P-40 non-ionic detergent, by three consecutive freeze–thaw cycles (liquid nitrogen–ice). After centrifugation for 30 min at 14,000 *g* at 4°, the supernatants were applied for the determination of dCK and thymidine kinase (TK) isoenzyme activities. Either ^{3}HdC or ^{3}HdT were used as substrates (both 10 μM; specific activities were between 500 and 1000 cpm/pmol), in a kinase assay buffer containing 50 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2 mM dithiothreitol, 10 mM NaF and as much cell extract (5 μL, corresponding to 25 μg of total protein) as to ensure that the reaction would proceed in the linear kinetic range. To stop the reaction, aliquots were spotted onto DEAE cellulose sheets, which were subsequently washed, dried, eluted and radioactivity was counted as described [15]. Enzyme activity was expressed as pmol phosphorylated deoxynucleoside per 10^6 cells per minute. In order to ascertain the contribution of TK2 to overall ^{3}HdC phosphorylation, excess concentration of non-labeled dT (1 mM) was included in the dCK reaction mixture [15]. The difference between ^{3}HdC phosphorylation measured in the presence and in the absence of 1 mM dT (TK2 activity) was only 1–2% of the total cellular dC phosphorylation capacity and remained constant during various treatments of cells.

2.4. Immunostaining of dCK

Cell extracts were prepared as described for the enzyme assays. Protein concentration was determined and 5 μL extracts (approximately 25 μg total protein) were resolved on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred to PVDF membranes (350 mAh) and stained with Ponceau S (0.1% (w/v) dye in 5% (v/v) acetic acid) to check equal loading. Membranes were blocked overnight in a buffer containing 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20 and 5% non-fat milk, then washed and probed with a highly specific antibody raised against the C-terminal domain of the dCK protein (epitope: ^{246}YESLVEKVKEFLSTL^{260}) [16] at 1:5000 dilution, followed by incubation with horseradish peroxidase conjugated anti-rabbit IgG (1:40,000). Immunocomplexes were visualized by the enhanced chemiluminescence reaction,

according to the manufacturer's instructions. Purification of dCK from human spleen and production of recombinant human dCK lacking the N-terminal 6xHis-tag were performed as described in [17].

Native Western blots were performed with the following changes as compared to denaturing blots: SDS and 1,4-dithio-L-threitol (DTT) were omitted from the standard 2× Laemmli loading buffer and samples were not boiled prior to loading; polyacrylamide gels were cast without SDS and the running buffer contained 25 mM Tris base and 192 mM glycine (pH 8.3); protein transfer was carried out with 2 mAh/cm^2 using a semi-dry blot apparatus in a transfer buffer composed of 25 mM Tris base and 192 mM glycine (pH 8.3).

For dot immunoblotting, nitrocellulose membranes were presoaked in 75 mM Tris–HCl pH 8.8 for 5 min, then protein samples were spotted and dried. Membranes were subsequently blocked and hybridized essentially the same way as described above.

2.5. Coomassie protein staining

Polyacrylamide gels were stained in a solution containing 50% methanol, 10% acetic acid and 0.2% (w/v) Coomassie Brilliant Blue R 250 at room temperature for 30 min with constant agitation. Destaining solution was composed of 50% methanol and 10% acetic acid.

3. Results

3.1. Disruption of dCK activation by selective depletion of intracellular but not extracellular Ca^{2+}

To demonstrate the hypothesized correlation between changes in the free cytosolic calcium levels and the function of dCK, the intracellular Ca^{2+} concentration was gradually diminished by treating short-term primary cultures of human tonsillar lymphocytes with increasing concentrations of the cell-permeable, selective Ca^{2+} chelator BAPTA-AM. BAPTA-AM is intracellularly activated by nonspecific esterases and the active form binds calcium ions with high affinity at a 1:1 molar ratio [14,18]. As depicted in Fig. 1A, BAPTA-AM inhibited both the control and the CdA-stimulated dCK activities in a concentration-dependent manner. The CdA-induced enzyme activity was significantly reduced even at very low concentrations (see the sharp decline from 231% to 192% at 1 μM BAPTA-AM in Fig. 1A). Co-incubation with 50 μM BAPTA-AM completely prevented the activation of dCK by CdA, therefore 50 μM BAPTA-AM was applied throughout the further experiments. Interestingly, BAPTA-AM caused an unexpected transient elevation of dCK activity at lower concentrations (up to 140% at 10 μM) followed by stepwise suppression in the higher concentration range (10–100 μM) where the two curves run in a parallel manner. BAPTA-AM was not toxic to the cells under

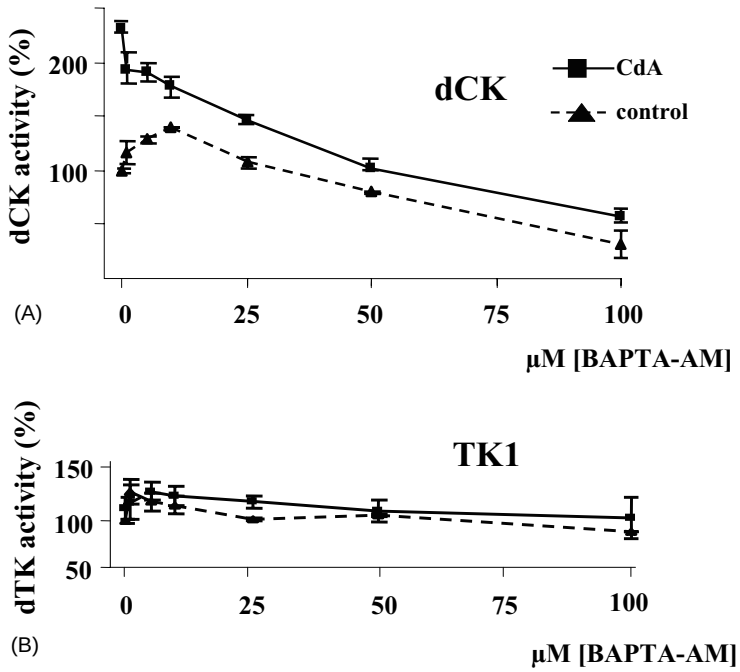

Fig. 1. Selective depletion of intracellular calcium results in decreased dCK activity. (A) Parallel cultures of human tonsillar lymphocytes were incubated with increasing concentrations of BAPTA-AM (range: 1–100 μM) in the presence (solid line) or absence (dashed line) of 2 μM CdA for 2 hr at 37°. dCK (A) and TK (B) activities were determined in the crude cell extracts with tritiated substrates as described in Section 2.3. and plotted as percentages of the untreated control. Control activities were 7.85 ± 0.08 pmol dCMP/10^6 cells/min for dCK and 3.59 ± 0.04 pmol dTMP/10^6 cells/min for TK1. Activity values are means of two parallels in two independent experiments; error bars represent the standard deviations.

our experimental conditions as proven by the trypan blue exclusion assay.

Recently, we have shown that dCK could also be activated in proliferating cells, including normal lymphocytes and different cell lines [9]. To explore whether BAPTA-AM could rescue the activation of dCK in dividing cells as well, we used primary cultures of human lymphocytes prepared from the thymus of 6- to 9-month-old children removed during cardiac surgery. Two-hour treatments with 50 μM BAPTA-AM reduced the enzyme activity to one fifth of the control value, and dCK activity was suppressed to the same level when cells were co-incubated with 2 μM CdA and 50 μM BAPTA-AM, as well.

In order to prove the specificity of the inhibitory effect of BAPTA-AM for dCK, we also measured thymidine kinase 1 (TK1) activities which showed no response to decreasing calcium concentrations (Fig. 1B). In the following experiment, the calcium concentration of the extracellular compartment was decreased by adding millimolar concentrations of EGTA, a highly hydrophilic compound, to the cell cultures. As it was expected, neither partial (1 mM EGTA) nor total (5 mM EGTA) depletion of the 1.8 mM Ca^{2+} in the cell culture medium had any significant effect on the dCK activity of the cells (data not shown).

We have recently proposed that dCK might exist in active and inactive forms in cells [12]. In the light of this, the question emerges whether BAPTA-AM prevents only the activation process itself or it also compromises the active state of the enzyme. To clarify this issue, we temporally dissected the inhibitory effect of BAPTA-AM on CdA-induced dCK activation. Lymphocytes were treated with 2 μM CdA alone for various time periods (0–120 min) and the obtained activation curve is displayed in Fig. 2 (solid line). Three independent cultures were incubated with 2 μM CdA for 2 hr, and 50 μM BAPTA-AM was added to the cells at 30, 60 and 90 min, respectively. dCK activities were measured at the end of the 2-hr incubation period and expressed as percentages of the untreated control (Fig. 2, dashed lines). BAPTA-AM significantly reduced dCK activities from the levels prior to the addition of the chelator, in spite of the continuous presence of CdA (e.g. from 255 to 125% if given at 60 min; see the declination of the dashed curves). This shows that addition of BAPTA-AM not only prevented CdA from fully activating the enzyme during the 2-hr incubation but also decreased the already achieved elevations in dCK activity. The observation that BAPTA-AM is less effective if given at 90 min, when only 30 min remained to exert its effect, can be partially attributed to the fact that intracellular cleavage of the prodrug by nonspecific esterases requires several minutes [14].

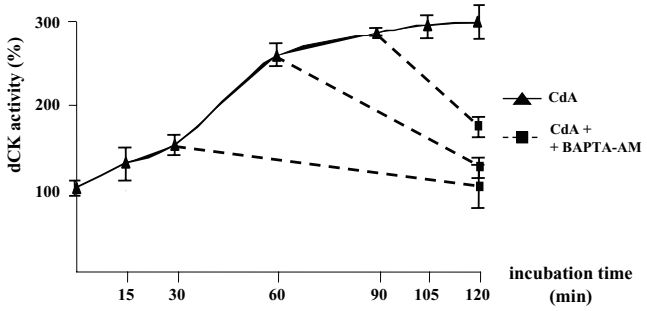

Fig. 2. Kinetics of the inhibitory effect of BAPTA-AM on CdA-induced dCK activation. Primary lymphocyte cultures were exposed to 2 μM CdA for the indicated time periods and dCK activities were subsequently measured (solid line). In the parallel experiments represented by the dashed lines, 2 μM CdA was present throughout the 120-min incubation, and cultures were complemented with 50 μM BAPTA-AM at the indicated time points (at 30, 60 and 90 min) followed by the enzyme assay at 120 min. All enzyme activities are expressed as percentage of the activity of the 120 min untreated control. Control dCK activity: 3.74 ± 0.07 pmol dCMP/10^6 cells/min.

3.2. Calcium plays a permissive role in the activation of dCK in cells

Having shown the inhibitory effect of calcium depletion on dCK activity in cells, we were prompted to check whether elevated intracellular calcium concentration itself could activate the enzyme. Thapsigargin, a potent inhibitor of the sarco-endoplasmic reticulum Ca^{2+} ATPase was used to release calcium from intracellular stores [19]. However, thapsigargin failed to activate dCK either in control or in CdA-stimulated cells (Fig. 3). The slight decrease in dCK activity at 2 mM thapsigargin could be explained by inhibition of the store-regulated transmembrane calcium fluxes that results in decreased cytoplasmic calcium levels [20], mimicking the effect of BAPTA-AM.

Similarly, no changes in dCK activity were obtained by using strophanthin, an inhibitor of the plasma membrane Na^+/K^+ ATPase that elevates intracellular calcium levels indirectly by increasing the inward transmembrane calcium

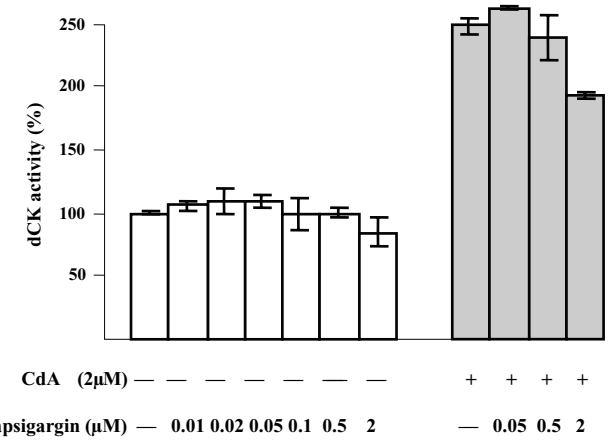

Fig. 3. Effect of thapsigargin on dCK activity in human lymphocytes. Primary cell cultures were maintained in Eagle's medium for 2 hr either in the presence (shaded columns) or in the absence of 2 μM CdA (blank columns) together with increasing concentrations of thapsigargin. dCK activities were determined in crude cell extracts as described in Section 2.3. Control dCK activity (100%): 3.32 ± 0.07 pmol dCMP/10^6 cells/min.

transport, or by ionomycin, a widely used calcium ionophore (data not shown). Taken together, these results ascribe a permissive role to calcium in the regulation of dCK, indicating that normal intracellular calcium levels are essential for the proper function of dCK but elevated calcium concentration does not activate the enzyme *per se*.

3.3. Calcium is not a cofactor of dCK

Calcium salts are not included either in the extraction buffer or in the kinase assay buffer, routinely used for measuring dCK activity (see Section 2.3). However, there is a theoretical possibility that dCK binds either Ca^{2+} ions or forms a complex with a calcium-binding protein in a structure that remains intact during cell extraction and preserves its enzyme activity under *in vitro* circumstances. To address this question, we included 50 μM BAPTA-AM in the dCK reaction mixture, supposing that BAPTA has much higher Ca^{2+}-binding affinity than the cellular proteins. BAPTA-AM had no effect on the dCK activity of either the control or the CdA-stimulated cell extracts. As expected, no changes were detected in the case of TK1, as well (data not shown).

To circumvent the possibility that cellular esterases might not function properly under the conditions of the kinase assay, and therefore BAPTA-AM might not be activated, it was replaced by EGTA with essentially the same results obtained (Fig. 4). These findings argue against the direct involvement of calcium ions in the sustenance of dCK activity.

3.4. Deoxycytidine kinase activation is accompanied by a conformational change

In order to explore whether inhibition of the enzyme activity is reflected by any changes in dCK protein levels,

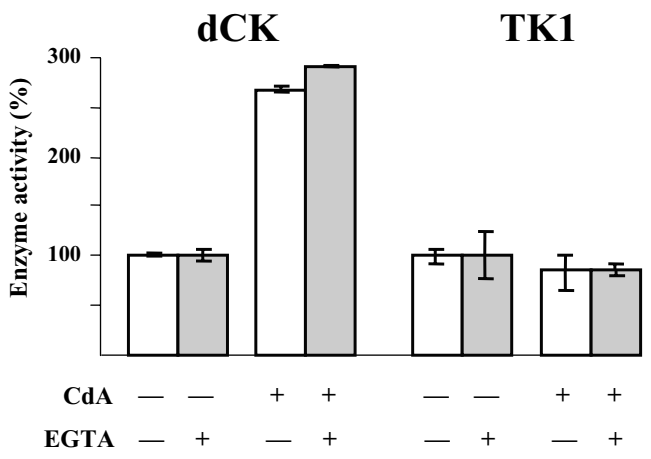

Fig. 4. Ca^{2+} is not required directly for the activity of dCK. Freeze–thaw whole cell extracts were prepared from control and 2 μM CdA-treated cultures of human tonsillar lymphocytes. dCK and TK1 activities of both extracts were measured in kinase reaction mixtures in the absence (blank columns) or in the presence of 500 μM EGTA (shaded columns), respectively. Control activities (100%): 3.09 ± 0.04 pmol dCMP/10^6 cells/min for dCK and 0.79 ± 0.05 pmol dTMP/10^6 cells/min for TK, respectively.

we performed denaturing immunoblots of extracts from BAPTA-AM-treated cells with a highly specific dCK antibody [16]. Figure 5A presents unambiguous evidence that this is not the case; cells treated with BAPTA-AM contain the same amount of dCK protein as those exposed to stimulating agents such as CdA or aphidicolin, despite several fold differences in enzyme activity levels which are on display beneath (Fig. 5D). As a positive control, different amounts of recombinant dCK protein were employed in the Western blot. Note that both the recombinant and the cellular dCK exhibit the same mobility since the N-terminal 6xHis-tag was removed by thrombin digestion from the bacterially expressed protein which would otherwise result in significant gel retardation [12,17].

Unexpectedly, a strikingly different picture emerged when the same cell extracts were run and blotted under non-denaturing conditions (Fig. 5B). The clear-cut uniformity of the bands disappeared and a direct correlation could be observed between the enzyme activity of dCK and the intensity of the corresponding bands on the membrane. dCK from BAPTA-AM-treated cells was poorly recognized by the antibody. Based on the denaturing Western blots, the same amount of dCK protein was found in the extracts. Therefore, we assume that the different staining pattern of the native immunoblots might be explained by the altered accessibility of the epitopes towards the antibody. It should be noted, however, that the exposition time of the denaturing immunoblot was much less than that of the native blot, implying an overall higher accessibility to the antibody in the completely denatured conformation of dCK (see also Fig. 6).

Surprisingly, the recombinant dCK could not be detected on the native blot. To prove that the appearing bands are specific dCK signals indeed, we ran 20 μg dCK protein purified from human spleen [17] on a native polyacrylamide gel and visualized it by Coomassie staining (Fig. 5C). Two bands could be seen corresponding to the dimeric and monomeric forms of dCK. Both ‘‘B’’ and ‘‘C’’ native gels were of the same acrylamide concentration and were run under the same conditions. Precise alignment of the two images revealed that the electrophoretic migration patterns of the Coomassie-stained native enzyme and the native immunoblots were identical, proving the specificity of the signals on the native blot. It is also interesting to note that there were no gross changes in the monomer/dimer ratio, irrespective of the different treatments of lymphocytes.

To understand the reason why the recombinant dCK did not give any signal on the native blot, we performed a dot blot immunoassay to compare the behavior of the recombinant and the purified human dCK species under native, reducing and denaturing conditions. Three aliquots of both preparations containing equal amounts of proteins were kept in Tris buffer (Fig. 6: ‘‘native’’), treated with 100 mM DTT (‘‘+DTT’’) or denatured aggressively by the combined action of heat, DTT and SDS (‘‘+SDS’’) as described

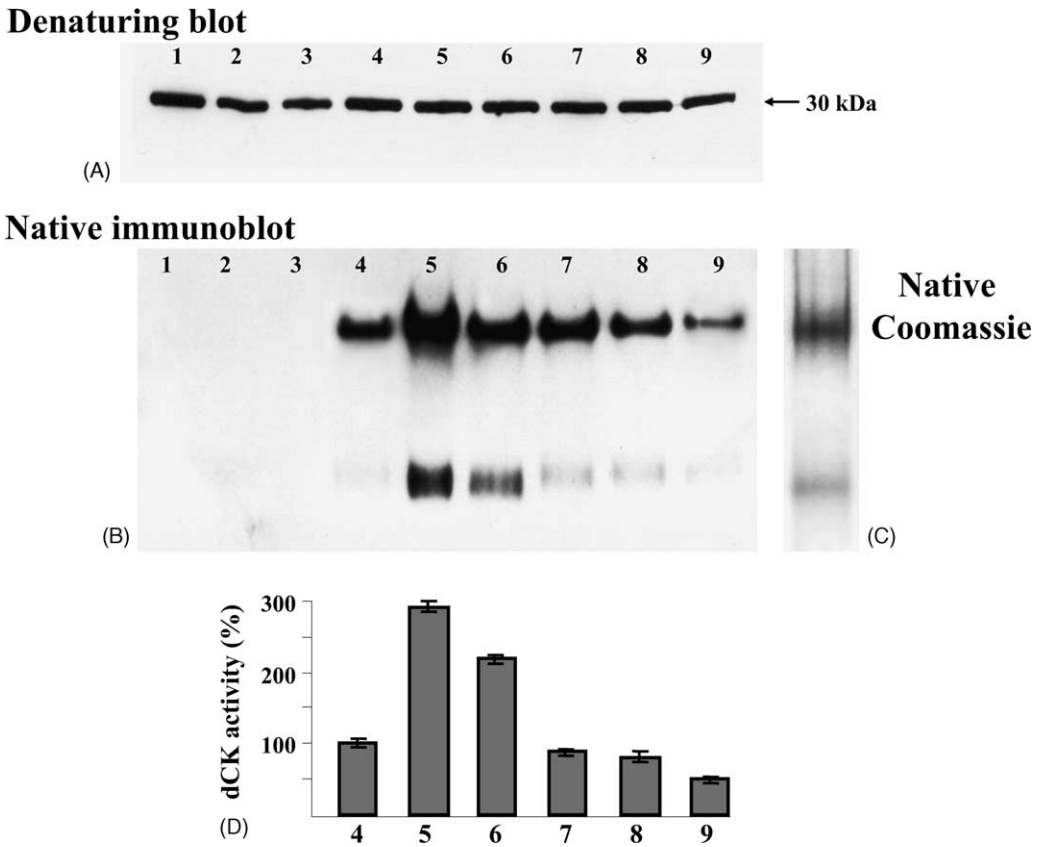

Fig. 5. Correlation between dCK enzyme activities and dCK protein levels detected by native Western blot. Lymphocytes were treated for 2 hr with different combinations of drugs as follows (numbers enlisted here correspond to the labeling applied in panel A, B and D): 4: control; 5: 2 μM CdA; 6: 2 μg/mL aphidicolin; 7: 2 μM CdA + 50 μM BAPTA-AM; 8: 2 μg/mL aphidicolin + 50 μM BAPTA-AM; 9: 50 μM BAPTA-AM. Following incubation, cells were washed, freeze–thaw extracts were prepared and protein concentrations were adjusted to 5 μg/μL. Twenty-five micrograms of total protein was run on 12% polyacrylamide gels followed by immunoblotting either under denaturing (denaturing blot; panel A, 15″ exposure) or native conditions (native blot; panel B, 5′ exposure) as described in Section 2.4. In lanes 1–3: 30, 20 and 10 ng recombinant dCK was applied, respectively. C: 20 μg human purified dCK was resolved on a 12% native polyacrylamide gel and stained with Coomassie Brilliant Blue. D: dCK enzyme activities were determined in the cellular extracts described above and displayed as percentages of the untreated control. Control activity (100%): 3.21 ± 0.09 pmol dCMP/10^6 cells/min.

in Fig. 6. Native and DTT-treated human dCK was equally well recognized by the antibody, while the maximally unfolded denatured protein gave the most intensive signal. In contrast, the recombinant protein was not recognized at all in the first two cases, but upon denaturation it bound the antibody with the same avidity as its counterpart of human origin did. In addition, the specific activity of the recombinant dCK preparation was 5-fold less than that of the human purified enzyme (data not shown). These results show that dCK might exist in different conformational states, exerting major impacts on its catalytic activity, as there seems to be a direct relationship between the enzyme

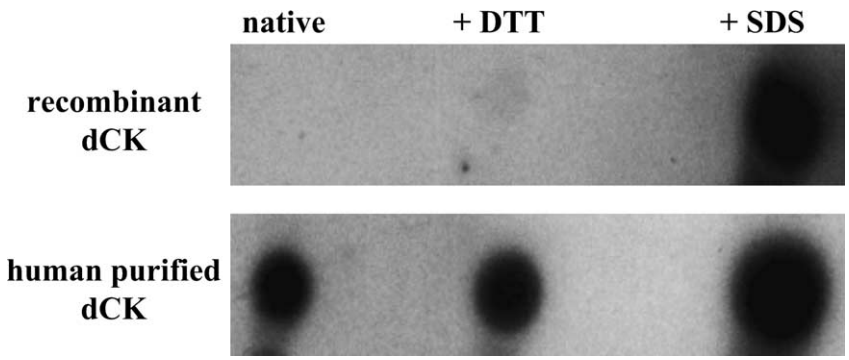

Fig. 6. Recombinant dCK is not recognized by the antibody under native conditions. Dot immunoblots were performed by spotting either 20 ng recombinant dCK (upper panel) or 20 ng human purified dCK (lower panel) on nitrocellulose membrane and probed with a specific antibody raised against the C-terminal peptide of dCK at a 1:5000 dilution. Protein samples were complemented with either 75 mM Tris–HCl pH 8.8 (native), 100 mM DTT (+DTT) or 2% SDS, 60 mM Tris–HCl pH 6.8 and 100 mM DTT (+SDS), respectively. SDS-containing samples were boiled for 2 min prior to spotting on the membrane.

activity and the epitope exposition to the antibody. The recombinant protein represents the most compact form that cannot interact with the antibody unless denatured.

4. Discussion

Unravelling the regulatory mechanisms controlling dCK activity possesses tremendous biomedical importance in the light of the central role played by dCK in the salvage of deoxyribonucleosides for DNA repair and in the activation of their analogues in chemotherapy [1].

In the present study we reported the inhibition of the activation process of dCK by depleting intracellular calcium pools. BAPTA-AM, a cell-permeable Ca^{2+} chelator totally rescued CdA-elicited dCK activation at 50 μM concentration. The same concentration of BAPTA-AM was previously shown to be sufficient for the complete abrogation of CdA-induced caspase 3-like activity and DNA fragmentation in CEM 0 cells [14], suggesting that dCK activation and apoptosis induction might be interrelated events indeed.

Notably, BAPTA-AM prevented not only the CdA-induced, but also the aphidicolin-, etoposide- and hydrogen peroxide-elicited elevations of dCK activity (data not presented). These data provide a strong indication that calcium might be a common factor in the various pathways converging to heighten the enzyme activity of dCK.

Extracellular calcium chelation had no effect on dCK activity. Moreover, inhibitors of transmembrane calcium uptake—the voltage-dependent calcium channel blockers nifedipine and verapamil and the nonvoltage-sensitive, stretch-activated cation channel blocker gadolinium (III) chloride—did not influence the enzyme, either (data not shown). These results imply that either the calcium homeostasis of lymphocytes is not disturbed substantially by short-term treatments with these drugs, or at least dCK activity is not sensitive enough to minor changes in free calcium levels. On the other hand, the failure to enhance dCK activity by treating lymphocytes with drugs that are known to elevate intracellular calcium concentration, such as thapsigargin, strophanthin, the calcium ionophore ionomycin, or depolarizing concentrations of KCl (data not shown), led us to assign a permissive rather than a decisive role to calcium in the context of dCK activation. Intracellular calcium concentration was already shown to possess such a modulatory function in various systems. A classical example is the inhibition of phospholipase C by calcium chelators and inorganic calcium blockers, while no elevation was obtained with either the Ca^{2+} ionophore A23187 or with depolarizing concentrations of K^+ [22].

A recent report, analogous to our findings, concerns the modulation of the GLUT1 glucose transporter by calcium [23], where the chelation of intracellular calcium with BAPTA-AM reduced sugar uptake in a dose-dependent fashion, with maximal inhibition at 75 μM. In addition, BAPTA-AM prevented the activation of GLUT1 by azide, arsenate, 2,4-dinitrophenol and insulin, while the elevation of cytosolic calcium concentration over the resting levels had no effect on basal sugar transport [23]. Calcium chelation did not change the abundance and distribution of GLUT1 in hepatocytes, similarly to our system where BAPTA-AM did not reduce dCK protein levels (Fig. 5A). A permissive role was also attributed to cytosolic calcium in the activation of the hepatic Na^+/taurocholate cotransporter through a Ca^{2+}/calmodulin complex formation [24] as well as in the regulation of mitogen-activated protein kinase via protein kinase C (PKC) upon angiotensin II signaling [25].

Our data demonstrated (see Fig. 4) that calcium is not an obligatory cofactor for dCK itself. It is equally unlikely that a dCK–Ca^{2+}/calmodulin complex or a dCK complex with any other calcium- or calmodulin-binding proteins would be involved in the formation of the catalytically active dCK, since this complex would be destroyed in the presence of calcium chelators in the crude extract. If calcium is not a direct effector of dCK, the question remains what its role in the activation process of dCK is. As previous data suggested that triggering of the transition of dCK from the inactive to the active state involved a protein phosphorylation step [12], we assume that calcium might participate in the regulation of a putative kinase which directly or indirectly (i.e. like an upstream member of a kinase cascade) phosphorylates and activates dCK. Whether this kinase binds Ca^{2+} such as the classical isoenzymes of PKC, or it is regulated by Ca^{2+}/calmodulin like the Ca^{2+}/calmodulin-dependent protein kinases remains to be investigated. The hypothesis assuming a calcium-dependent phosphorylation event is consistent with the kinetic data presented in Fig. 2, postulating that calcium is essential for the maintenance of the active state. If the putative kinase activity is crippled by the removal of calcium, cellular protein phosphatases could dephosphorylate and thereby inactivate dCK. The importance of protein phosphatases in the inactivation of dCK has indirectly been proven by the protein phosphatase 1 and 2A inhibitor calyculin A [12].

There are several consensus phosphorylation sites for PKC in the sequence of dCK, and PKCα has already been shown to phosphorylate and activate dCK *in vitro* [26]. On the other hand, we have shown that phosphorylation by PKCα did not alter the activity of the recombinant dCK [27]. Therefore, the potential role of PKC in the activation of dCK is ambiguous.

In accordance with previous results indicating constant protein levels of dCK upon various short-term treatments [8,10–12], we could detect no changes in the amount of dCK protein in BAPTA-AM-treated cell extracts by denaturing Western blots (Fig. 5A). In sharp contrast to this, the native blot disclosed huge differences between the staining intensity of the same extracts; moreover, the recombinant control did not give any signal under native conditions.

Two possible explanations could be conceived. First, the recombinant dCK did not migrate into the gel or was not transferred to the membrane; or second, it was present on the membrane but was not recognized by the antibody due to its compact conformation under native conditions. Although the first possibility seemed improbable since the isoelectric point of the protein is well below 7 and the electrophoresis and blotting were carried out at pH 8.3, we performed a dot blot that verified the second scenario. These results provide further justification of the 'inactive-active transition theory' by direct visualization of the different conformations assumed by the catalytically 'active' and 'inactive' forms of the enzyme. The recombinant enzyme is free from secondary modifications and adopts a closed conformation where the epitope is masked and inaccessible. The eukaryotic counterpart might be more open and the epitope becomes accessible to the antibody under native conditions. dCK might exist in two different forms in cells whose actual balance shows parallel changes with enzyme activation/inactivation. The relative proportion of the 'more active' (i.e. modified and opened) counterpart was increased upon CdA- or aphidicolin-treatments, manifested by elevated enzyme activity and more intensive signals on the native blots. The unmodified ('inactive') variant was not visible on the native blot. It must have, however, some basal enzyme activity since recombinant dCK also possesses kinase activity [28].

Our dCK antibody was raised by immunizing rabbits with a 15-mer peptide corresponding to the very C-terminal region of the human dCK [16]. The recent resolution of the crystal structure of recombinant human dCK has shown that this C-terminal epitope is embedded within the 19-mer α10 helix of the protein, which is located opposite to the dimer interface composed of the α4/α7 four-helix bundle [21]. Helix α10 is joined to the central β5 sheet by a short linker peptide. One may speculate that this linker region is flexible enough to behave as a hinge, allowing the helix to jut out from the surface of the globular protein upon local or global conformational changes of dCK, providing better accessibility for the antibody.

Mono- and polyclonal antibodies are versatile tools for studying protein structure. Conformational changes often lead to drastic changes in antibody accessibility. For instance, a specific anti-progesterone receptor antibody recognizes the protein only if it is bound to an antagonist ligand, but it does not recognize the receptor bound to its agonists [29]. An antipeptide antibody directed against the kinase domain of the insulin-like growth factor receptor I immunoprecipitated the phosphorylated form, but did not react with the nonphosphorylated form of the protein. In denaturing Western blots, however, the antibody identified equally well both forms of the receptor due to complete unfolding—similarly to our observations concerning dCK [30]. There are other indications as well for antibodies showing reduced affinity towards the dephosphorylated forms of certain proteins [31,32].

It is important to note that the results presented here make two other potential scenarios of dCK activation rather improbable. The active form of dCK is the homodimer [33], which might suggest a possible regulation through homodimerization, as it was found in other cases [34]. The dominant form of dCK was the dimer on the native blot and no conspicuous monomer/dimer transitions were detected despite gross changes in the enzyme activity (Fig. 5B and D), calling into question the possibility of increased formation of homodimers as a potential mechanism of enzyme activation. Redox-sensitive conformational changes might also provide regulatory mechanisms [35,36], and dCK harbours six cysteine residues. However, no changes were detected in the dot blot assay upon addition of 100 mM DTT in case of either the recombinant or the human purified dCK (Fig. 6) that strongly argues against the possible role of disulfide bond formation in the regulation of the conformation of this enzyme.

In summary, two important aspects of the activation of dCK have been revealed in this paper: (1) the permissive role of calcium ions and (2) the conformational change of the enzyme upon activation. These results underpin our previous suggestion regarding the post-translational modification of the enzyme during activation. The clear-cut distinction between the two structural forms of the enzyme was achieved by native immunostaining. However, further investigations are needed to decipher the molecular details of dCK activation.

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