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A crucial role for ATR in the regulation of deoxycytidine kinase activity

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ABSTRACT

Deoxycytidine kinase (dCK) (EC 2.7.1.74) is a key enzyme for salvage of deoxynucleosides and activation of numerous anticancer and antiviral nucleoside analogs. dCK activity is enhanced in response to several genotoxic treatments, which has been correlated with an increase of dCK phosphorylation at Ser-74. ATM was recently identified as the kinase responsible for Ser-74 phosphorylation and dCK activation after ionizing radiation (IR). Here, we investigated the role of ATM and the related kinase ATR in dCK activation induced by other types of DNA damage. Using ATM-deficient cells or the ATM inhibitor KU-60019, we found that ATM was not required for dCK activation caused by UV light, aphidicolin, cladribine, and unexpectedly also IR. On the other hand, the selective ATR inhibitor VE-821 significantly reduced up-regulation of dCK activity induced by these genotoxic agents, though not IR, and also down-regulated basal dCK activity. A role for ATR in the control of dCK activity was confirmed by using ATR siRNA and ATR-Seckel cells. ATR was also found to directly phosphorylate dCK at Ser-74 *in vitro*. Further studies revealed that ATR, which is also activated in response to IR, although later than ATM, was responsible for IR-induced dCK activation in ATM-deficient cells or in the presence of KU-60019. Overall, our results demonstrate that ATR controls basal dCK activity and dCK activation in response to replication stress and indicate that ATR can activate dCK after IR if ATM is lacking or inhibited.

1. Introduction

Deoxycytidine kinase (dCK) catalyzes the phosphorylation of deoxycytidine, deoxyadenosine and deoxyguanosine into their monophosphate form. This reaction is the first and rate-limiting step of the deoxynucleoside salvage pathway that supplies cells with deoxyribonucleotides for DNA synthesis as an alternative to the *de novo* synthesis [1]. In addition to its natural substrates, dCK phosphorylates and activates a large number of nucleoside analogs used in the treatment of cancer and viral diseases [2,3], thereby playing an essential role in their therapeutic efficacy [4].

Given the important role of dCK in deoxynucleotide metabolism and in human chemotherapy, identification of the mechanisms that control its activity is of highest interest. We previously established that dCK is a phosphoprotein, containing at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue [5]. Site-directed mutagenesis demonstrated that Ser-74 phosphorylation increases basal dCK activity, whereas phosphorylation of the three other sites does not [5,6]. In addition, the use of a specific anti-phospho-Ser-74 antibody showed that activation of dCK, which is observed in response to a series of genotoxic treatments, including ionizing radiation (IR), UV-C light, DNA synthesis inhibitors and chemotherapeutic nucleoside analogs [7–12], is correlated with an increase of the phosphorylation of dCK at Ser-74 [5,13].

Next step was to decipher the signaling pathway involved in the control of Ser-74 phosphorylation. The finding that dCK was activated by genotoxic stimuli suggested that a DNA damage-activated protein kinase could be involved in this process. In accordance with this hypothesis and the results from a global proteomic analysis [14], the ATM (ataxia-telangiectasia mutated) kinase, a master regulator of the DNA damage response, was identified as the protein kinase responsible for Ser-74 phosphorylation and dCK activation in response to IR [15,16]. Concerning dCK dephosphorylation, we recently showed that protein phosphatase 2A (PP2A) constitutively dephosphorylates Ser-74 and is therefore

Abbreviations: APC, aphidicolin; ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad-3 related; CdA, cladribine; dCK, deoxycytidine kinase; DSB, double-strand break; IR, ionizing radiation; PP2A, protein phosphatase 2A; siRNA, small interfering RNA; ssDNA, single-stranded DNA.

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a negative regulator of dCK activity [17]. Interestingly, we noted in this study that the PP2A inhibitor okadaic acid induced increase of Ser-74 phosphorylation and dCK activity in ATM-deficient cells, suggesting that another kinase than ATM was able to phosphorylate dCK at Ser-74 in basal conditions [17].

Whereas IR predominantly activates the kinase ATM, other genotoxic agents known to increase dCK activity, such as UV-C light and the DNA polymerase inhibitor aphidicolin, primarily activate ATR (ATM and Rad-3 related), another essential kinase involved in the DNA damage response. Both ATM and ATR promote cell cycle arrest and DNA repair or induce apoptosis if repair systems are overwhelmed [18], but they respond to distinct DNA lesions: ATM is activated in response to DNA double-strand breaks (DSBs), such as induced by IR, while ATR is activated by single-stranded DNA (ssDNA) [19]. This ssDNA structure is generated during processing of DSB or arises when DNA replication forks stall, which can occur during normal replication (for instance at fragile sites) or in response to DNA synthesis inhibitors, chemotherapeutic drugs or UV irradiation [20,21]. Like ATM, ATR phosphorylates its targets on Ser or Thr residues that are followed by Gln (SQ/TQ motifs) [14], as is Ser-74 of dCK, suggesting that ATR could phosphorylate Ser-74 and activate dCK in response to certain genotoxic stimuli.

In the present study, we aimed to compare and investigate the roles of both ATM and ATR in Ser-74 phosphorylation and dCK activation in response to various DNA-damaging agents, and particularly to UV light and genotoxic drugs that cause replication stress. Our results show that ATR, besides ATM, plays an important role in the regulation of dCK activity, not only after DNA damage, but also in unperturbed cells.

2. Materials and methods

2.1. Reagents

RPMI-1640 and all cell culture reagents were from Gibco/Invitrogen (Carlsbad, CA, USA). Fetal calf serum (FCS) and ultraglutamine were purchased from Lonza (Basel, Switzerland). Cladribine (2-chloro-2′-deoxyadenosine, CdA) was synthesized and supplied by Prof J. Marchand (Laboratory of Organic Chemistry, Université catholique de Louvain, Louvain-la-Neuve, Belgium). [5-^{3}H]-deoxycytidine (18 Ci/mmol) was from Moravek Biochemicals (Brea, CA, USA). KU-60019 and VE-821 were purchased from Bio-Connect (Huissen, The Netherlands). The protein A/G PLUS-agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals, materials and reagents were from Sigma, Calbiochem or Bio-Rad Laboratories.

2.2. Cell culture and treatments

The EBV-immortalized lymphoblastoid cell lines GM0536 ($ATM^{+/+}$), referred here as wild-type cells, and GM1526 ($ATM^{-/-}$), which were derived from a healthy control or an ataxia telangiectasia patient, respectively, were obtained from the NIGMS Human Mutant Cell Repository (Camden, NJ, USA). The Seckel lymphoblastoid cell line GM18367A was kindly provided by Dr. X. Liu (Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA). The human leukemia cell lines EHEB (chronic B-cell leukemia) and HL-60 (acute myeloid leukemia) were purchased from DSMZ-German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). MCF-7 (breast) and PANC-1 (pancreatic) cancer cell lines were kind gifts from Dr. A. Decottignies and Dr. G. Bommer (de Duve Institute), respectively. GM0536, GM1526 and GM18367A cells were cultured in RPMI-1640 medium with Glutamax supplemented with 15% FCS at 37 °C in humidified air containing 5% CO_2. EHEB and HL-60 cells were cultured in the same conditions, except that FCS was used at a concentration of 10%. MCF-7 and PANC-1 cells were cultured in DMEM medium supplemented with 10% FCS, 2 mM ultraglutamine and penicillin-streptomycin (100 U/ml).

When used, inhibitors were added 1 h before genotoxic treatment. Hydrophobic compounds were dissolved in DMSO and equal amounts of DMSO were added in untreated and treated cells. The final DMSO concentration was ≤0.2%. In some experiments, cells were UV-C-irradiated as described in [7] or submitted to IR using a ^{137}Cs source at a dose rate of 2.43 Gy/min at room temperature.

2.3. dCK assay in cell lysates

GM0536, GM1526, GM18367A, EHEB, HL-60, MCF-7 and PANC-1 cell extracts were prepared as previously reported [5,12]. dCK activity was measured by a radiochemical assay as described in [5], using 30–100 μg of cellular protein or 0.25 μg of recombinant dCK, with 10 μM [5-^{3}H]-deoxycytidine (~1000 cpm/pmol) and 5 mM Mg-ATP as substrates. The protein content of samples was determined by the method of Bradford, using BSA as a standard [22]. To facilitate the comparison between the different experimental conditions and cell lines, dCK activities were expressed as fold change. Basal dCK activities in wild-type (GM0536) and $ATM^{-/-}$ (GM1526) cells were 13.5 ± 1.0 ($n = 18$) and 11.1 ± 1.6 ($n = 9$) pmol/min/mg protein, respectively. For other cell lines, dCK activities are given in the legend of the figures.

2.4. Immunoblot analysis

Aliquots of cell lysates containing 30–150 μg of protein or 0.25 μg of recombinant dCK were subjected to SDS-PAGE in 12% (w/v) polyacrylamide gels and transferred to Immobilon-P Transfer membranes (PVDF) (Millipore, Billerica, USA). After transfer, the membranes were blocked at room temperature for 1 h in Odyssey blocking buffer, or in PBS or TBS containing 5% (w/v) fat-free milk powder or BSA, and then probed overnight at 4 °C with primary antibodies. After extensive washing in either PBS-T or TBS-T, the membranes were incubated with the secondary antibody at room temperature for 1 h. After washing, the membranes were scanned with the Odyssey Infrared Imaging System from LI-COR Biosciences (Nebraska, NE, USA) and fluorescence intensities were used to quantify dCK expression or phosphorylation [6]. Other proteins analyzed in this work were visualized using the Clarity™ Western ECL Substrate from Bio-Rad (Hercules, CA, USA) and band intensities were calculated using the image J software. Phosphorylations of dCK, Chk1 and Chk2 were normalized to dCK, Chk1 and Chk2 protein levels, respectively. Mean values obtained from three independent experiments are given under a representative Western blot image, the values found in untreated cells being set at 1 for comparison.

Primary antibodies used in this study were: anti-ATR (sc-1887) and anti-p53 (sc-126) from Santa Cruz Biotechnology, anti-p53-pS15 (9284L), anti-ATM (2873S), anti-ATM-pS1981 (5883S), anti-Chk1 (2360S), anti-Chk1-pS317 (2344S), anti-Chk2 (2662S), anti-Chk2-pT68 (2197S) from Cell Signaling Technologies (Beverly, MA, USA) and anti-β-actin (A5441) from Sigma–Aldrich (St. Louis, MO, USA). Anti-phospho-Ser-74 and anti-dCK antibodies were generated as previously described [5]. Anti-poly(His) antibody used to detect recombinant dCK was from GE Healtcare (Machelen, Belgium). Secondary antibodies were from Sigma–Aldrich (anti-rabbit and anti-mouse IG conjugated to horseradish peroxidase) or from Westburg (Leusden, The Netherlands) (IRDye® 800CW donkey anti-goat, IRDye® 800CW goat anti-rabbit and IRDye® 680 goat anti-mouse).