The Intracellular Localization of Deoxycytidine Kinase*

Pantelis Hatzis†, Ashraf Said Al-Madhoon‡, Mia Jüllig§, Thodoris G. Petrakis†, Staffan Eriksson§, and Iannis Talianidis‡‡

From the †Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, 711 10 Herakleion, Crete, Greece and ‡Department of Veterinary Medical Chemistry, The Biomedical Center, S-751 23 Uppsala, Sweden

Deoxycytidine kinase (dCK) catalyzes the rate-limiting step of the deoxynucleoside salvage pathway in mammalian cells and plays a key role in the activation of several pharmacologically important nucleoside analogs. Using a highly specific polyclonal antibody raised against a C-terminal peptide of the human dCK, we analyzed its subcellular localization by Western blots of biochemically fractionated nuclear and cytoplasmic fractions as well as by in situ immunocytochemistry. Native dCK was found to be located mainly in the cytoplasm in several cell types, and the enzyme was more concentrated in the perinuclear and cellular membrane area. In contrast, when dCK was overexpressed in the cells, it was mainly located in the nucleus. The results demonstrate that native dCK is a cytoplasmic enzyme. However, it has the ability to enter the nucleus under certain conditions, suggesting the existence of a cytoplasmic retention mechanism that may have an important function in the regulation of the deoxynucleoside salvage pathway.

Deoxycytidine kinase (dCK) catalyzes the phosphorylation of 2′-deoxycytidine to its monophosphate form, which is a rate-limiting reaction of the deoxynucleoside salvage pathway (1, 2). Studies with purified human enzyme have demonstrated that dCK has a broad substrate specificity using both purine and pyrimidine deoxynucleoside substrates (2–4). dCK is responsible for the initial activation of a number of clinically important anticancer and antiviral drugs, such as 2-chloro-2′-deoxyadenosine, 1-β-D-arabinofuranosylcytosine (araC), and 2′,2′-difluorodeoxycytidine (gemcitabine) (3–7). Impaired dCK expression or activity in cells usually leads to resistance for these drugs, indicating that dCK plays key role in their metabolism and pharmacological activities (8–10).

For these reasons, elucidation of potential regulatory mechanisms playing a role in dCK activation is of particular interest. Although much is known about the biochemical properties of dCK both in vitro and in vivo, little information is available on its regulation at the transcriptional or posttranslational levels. The constant amounts of dCK protein throughout the cell cycle suggest that its expression is not cell cycle-regulated (1, 11). On the other hand, dCK is expressed predominantly in lymphoid cells, which indicates cell type-specific regulation (12–15). In addition, dCK expression is up-regulated in certain solid tumors (16). The molecular mechanism that leads to tissue-specific and proliferation-dependent transcription of dCK is not yet clear.

Another level of control would involve regulated changes in the subcellular location of the enzyme. This assumption gained ground by the recent unexpected finding that a transfected dCK-green fluorescent protein (GFP) fusion protein was located mainly in the nucleus, contrasting with previous descriptions of dCK as a “cytoplasmic” protein (17). This finding may have important implications for the regulation of the deoxynucleotide metabolism, especially with respect to the functional compartmentalization of the dNTP pools. The determination of the actual location of native dCK is therefore of paramount interest.

In this paper we examined the intracellular localization of dCK in several cell types under physiological conditions. Using a highly specific C-terminal peptide antibody, we provide evidence that dCK is normally located in the cytoplasm. On the other hand, when dCK is overexpressed, it is found mainly in the nucleus, suggesting that a cytoplasmic retention regulatory mechanism may be functioning in living cells.

MATERIALS AND METHODS

dCK Antiserum Production and Immunoblot Assays—A peptide (NH2-YESLVEKYLSTL-COOH) was synthesized that corresponded to amino acids 246–260 of the human dCK protein (12), linked to keyhole limpet hemocyanin via maleimidobenzoyl-imido ester, pH 7.0, as described (18). New Zealand White rabbits were injected subcutaneously with 0.1 mg of linked peptide in complete Freund’s adjuvant and boosted five times with 0.04 mg of antigen in incomplete Freund’s adjuvant at 2-week intervals. Antisera were collected and tested in Western blots containing various amounts of purified recombinant His-tagged human dCK (15), and whole cell extracts from CCRF-CEM cells or the araC-resistant, dCK-deficient cell line CEM− (AraC-SD (10)). For immunoblot analysis, proteins were separated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the dCK-pep antibody at 1:5000 dilution, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson Laboratories). Immunocomplexes were visualized by the enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech). The polyclonal antiserum for the P1 protein (TSG23, a kind gift from C. Hoog, Karolinska Institute) and the monoclonal antibody for the myc epitope (9E10 hybridoma, kindly provided by N. T. Kitisakis, Babraham Institute) were used at 1:4000 and 1:1000 dilutions, respectively.

Isolation of Subcellular Fractions—Preparations of nuclear and cytoplasmic extracts were carried out essentially as described (19), with some minor modifications. Briefly, 1–5 × 106 cells were resuspended in isosmotic lysis buffer containing 0.32 m sucrose, 3 mM CaCl2, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM...
dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% (v/v) Nonidet P-40. The lysates were centrifuged at 500 × g for 5 min, and the supernatants were supplemented with 0.22 volume of cytoplasmic extract buffer containing 0.15 M HEPES, pH 7.9, 0.7 M KCl, and 0.015 M MgCl₂. After centrifugation at 12,000 × g for 15 min, the resulting supernatants were supplemented with glycerol to 25% and stored at −70 °C. The nuclear pellet was washed once in isosmotic lysis buffer lacking Nonidet P-40 and incubated for 30 min at 37 °C in a modified electrophoresis sample buffer containing 22% glycerol, 125 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 1% SDS, 0.01% bromphenol blue, 5 mM MgCl₂, and 75 units/ml Benzonase (Nicolm Pharma A/S). Before loading on the gel, samples were diluted with an equal volume of sample buffer without MgCl₂ and Benzonase.

Immunodepletion and Enzyme Activity Assays—100 μl (50% suspension) of protein G-Sepharose (Amersham Pharmacia Biotech) beads were incubated with dCK pep antisera or preimmune serum (100 μl of antiserum/mg of dry gel) for 1 h at 4 °C, in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM NaF, 1 mM EDTA, and 0.5% Nonidet P-40. The beads were washed with excess buffer and incubated with crude extracts from 5 × 10⁶ CCRF-CEM cells for 1 h. At the end of incubation, the beads were removed by centrifugation, and deoxycytidine kinase and thymidine kinase activities of the resulting supernatants were measured by the DEAE-cellulose filter binding assay as described previously (16, 20).

Cell Culture and Transfections—A2780, HeLa, and CCRF-CEM cells were grown in Dulbecco’s modified Eagle’s Medium and RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The A2780, HeLa, and CCRF-CEM cells were transfected by calcium phosphate coprecipitation and Lipofectin (Life Technologies, Inc.) method (15) into the CCRF-CEM cells by the calcium phosphate coprecipitation method or CCRF-CEM cells by the Lipofectin method (Life Technologies, Inc.) or 25% and stored at

To study the intracellular localization of dCK, we first performed biochemical fractionation of cytoplasts and nuclei from three cell types of different origin, such as the human malignant T lymphoblastoid leukemia (CCRF-CEM), human ovarian carcinoma (A2780), and human cervical carcinoma (HeLa) cell lines. We used an isosmotic/Nonidet P-40 lysis procedure (19) as an alternative to the commonly used hypotonic/Nonidet P-40 method to isolate intact nuclei and accurately compare the nuclear and cytoplasmic contents of dCK. Immunoblot analysis of these extracts showed that the bulk of cellular dCK protein was in the cytoplasmic fractions, whereas nuclei contained only a minor portion of the protein (Fig. 2A). To exclude the possibility of potential leakage of dCK from the nuclei to the cytoplasm during the fractionation procedure, we examined the distribution of the nuclear protein P1. P1 is a 105-kDa protein that is present only in the nucleus throughout the cell cycle in mammalian cells (23). We chose as a control P1 because it is a “free-floating” protein, not associated tightly with chromatin or structural components of the nucleas, making it a reliable marker for assaying nuclear protein leakage (23). Two strong bands at ~105 kDa were detected in the nuclear fraction of A2780 cells with P1 antisera (Fig. 2B). Only trace amounts of these bands were seen in the cytoplasmic fractions, together with some smaller cross-reactive species (Fig. 2B), indicating that the integrity of nuclei during the fractionation procedure has been preserved to a high degree.

The above results suggest that dCK is a cytoplasmic protein. This is in contradiction with the findings of a previous study using a dCK-GFP fusion protein, which was located mainly in
most exclusively in the nucleus in all three cell lines, as evidenced by the similar staining pattern obtained by the myc-tag antibody (Fig. 4, A–C) and the control antibody recognizing the nuclear protein Sp-1 (Fig. 4, D–F). The same distribution was observed when the cells were transfected with an untagged expression vector and stained with dCK-pep antibody (data not shown), excluding the possibility of artificial translocation of dCK from the cytoplasm to the nucleus as a result of the short myc epitope at the N terminus of the protein. Taken together, these results demonstrate that the nuclear localization of dCK in transfected cells is a consequence of its highly elevated intracellular levels.

**DISCUSSION**

In mammalian cells deoxyribonucleotides for nuclear DNA replication and repair are synthesized via the *de novo* pathway, which involves reduction of ribonucleotide diphosphates by ribonucleotide reductase, or via direct phosphorylation of deoxyribonucleosides imported from the extracellular space or derived from dephosphorylation of dNTPs (24). Earlier studies trying to explain the functional link between dNTP precursor metabolism and DNA replication processes showed a physical association of DNA polymerase α, with several key enzymes involved deoxynucleotide metabolism (25). The “repliplate model” has since been challenged by several observations, such as the findings that the enzymes thought to be components of the multienzyme complex were localized in different cellular compartments (26, 27). In addition, a number of kinetic isotope incorporation experiments argue against the direct channeling of precursors to the sites of DNA replication (28). These studies led to the prevailing view that dNTPs are synthesized in the cytoplasm and subsequently translocate to the nucleus to participate in DNA replication or repair. The recent finding that a GFP-dCK fusion protein was located in the nucleus raised a potentially interesting exception to the above rule (17).

However, the possibility that native dCK is an exclusively nuclear protein generates a number of questions. Although this location would favor the direct supply of dCTP for DNA replication, it is difficult to explain certain results from kinetic isotope incorporation experiments. These studies showed the existence of two dCTP pools: one labeled mainly via the *de novo* pathway and used directly for DNA replication, and the other labeled via the salvage pathway and preferentially used for liponucleotide synthesis (29–32). This second “cytoplasmic” dCTP pool is in rapid equilibrium with the one arising from the *de novo* synthesis and can be used for DNA synthesis efficiently (28–32). It is, however, difficult to explain why the *de novo* pool, which is the primary source of nuclear DNA synthesis precursors, is built up by cytoplasmic enzymes such as ribonucleotide reductase, whereas the salvage pool, which has to equilibrate first with the *de novo* pool to be used for DNA synthesis, would be synthesized by an enzyme located in the nucleus. In addition, a nuclear dCK as a rate-limiting enzyme for the dCTP supply of liponucleotides is not compatible with the rapid labeling of these phospholipid precursors from deoxycytidine (29–32), even if one takes into account a free passage of dCTP across the nuclear membrane.

The results presented in this paper clearly establish that native dCK is mainly located in the cytoplasm, a location more consistent with the previously mentioned metabolic studies. This conclusion is based on two independent approaches using biochemical cell fractionation and *in situ* immunochemistry experiments. A cytoplasmic localization was seen in three cell lines of different origin, as well as in primary thymocytes and splenocytes. In adherent cells, such as A2780 and HeLa, where the intracytoplasmic distribution is better resolved, we noticed denser staining of the cytoplasmic membrane and the perinu-

**FIG. 2.** Distribution of dCK in biochemically fractionated cellular compartments. A, cytoplasmic (C) and nuclear (N) extracts from CCRF-CEM (CEM), A2780, and HeLa cells were prepared and analyzed on Western blots by dCK-pep antiserum. B, immunoblot of cytoplasmic (C) and nuclear (N) extracts from A2780 cells by the antibody recognizing the P1 nuclear protein. C, CCRF-CEM (CEM), A2780, and HeLa cells were transfected with pCMV-myc-dCK plasmid, and cytoplasmic (C) and nuclear (N) extracts were prepared and analyzed on Western blots using the myc-tag antibody. Control lanes represent extracts from untransfected A2780 cells (Contr.).
It is tempting to speculate that this uneven distribution of dCK may be of functional relevance, corresponding to a compartmentalization that is related to the dCTP supplies for membrane phospholipid precursor synthesis and DNA replication, respectively. Alternatively, these sites may represent independent compartments for the phosphorylation of exogenous deoxycytidine immediately after its transport into the cell and deoxycytidine derived from endogenous dCTP breakdown, respectively. To resolve the contradiction between the localization of the GFP-dCK fusion protein (17) and the localization of the endogenous native protein, we asked whether the nuclear location of the former is attributable to its overexpression in the cells. We found that this is indeed the case, because after transfection of an expression vector coding for human dCK, which results in higher intracellular concentrations of dCK by several orders of magnitude, the overexpressed protein was mainly localized in the nucleus in all cell types tested. This finding strongly suggests that dCK has the ability to enter the nucleus. Further evidence for this notion was provided by the identification of a consensus nuclear import sequence at the N-
terminus of the protein, the mutation of which prevents nuclear entry even of the overexpressed GFP-dCK fusion protein (17).

Taking these findings together, we propose a cytoplasmic retention mechanism for dCK, which may involve the action of another protein complexed with dCK, that keeps it in the cytoplasm. When dCK is overexpressed, because of the limiting amounts of the cytoplasmic retention partner, the majority of dCK molecules will contain an accessible NLS sequence for recognition by the nuclear transport machinery. An analogous situation has been described for nuclear factor κB (33, 34), the regulation of which involves cytokine-induced phosphorylation of its cytoplasmic retention partner, IκB, which leads to the dissociation of the nuclear factor κB/IκB complex and the translocation of active nuclear factor κB subunits into the nucleus. An alternative model would involve posttranslational modification of dCK itself, which may be required for nuclear entry in analogy to the transcription factors involved in the interferon signaling pathways (35). Although at this point the actual mechanism that retains dCK in the cytoplasm is not known, our results raise the interesting possibility that under certain physiological conditions transport of dCK between subcellular compartments may play an important role in the regulation of its known function in the supply of deoxynucleotides for different biological processes or for unknown additional functions of the protein.

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