Identification of Phosphorylation Sites on Human Deoxycytidine Kinase Overexpressed in HEK 293T Cells and Purified Using TALON® Resin

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Several lines of evidence have indicated that human deoxycytidine kinase (dCK) may be a phosphoprotein, but this has not been demonstrated directly. To verify this hypothesis, dCK was overexpressed in HEK 293T cells with an N-terminal polyhistidine tag, allowing purification with TALON Metal Affinity Resin. After incubating the cells with [32P]-orthophosphate, dCK was purified, subjected to SDS-PAGE and autoradiography, and found to be [32P]-labeled. Tryptic digestion of purified dCK and analysis of the radioactive peptides by tandem mass spectrometry led to the identification of four in vivo phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser-74, the last being the most heavily phosphorylated. This study provides direct evidence that dCK is constitutively phosphorylated in eukaryotic cells.

Introduction

Deoxycytidine kinase (dCK) catalyzes the rate-limiting step in the deoxynucleoside salvage pathway. It also initiates the activation of several nucleoside analogues commonly used in anticancer and antiviral chemotherapy and plays an essential role in their therapeutic efficacy (1). Recent evidence suggests that dCK activity may be regulated by posttranslational modification. An increase in dCK activity, which cannot be explained by a change in the dCK protein level, has been repeatedly observed in normal or leukemic lymphocytes after treatment with genotoxic agents (2, 3). Moreover, treatment of cell extracts with λ-protein phosphatase decreased dCK activity (2, 3). Our study was initiated to verify that dCK is phosphorylated in intact cells (4).

In vivo phosphorylation of dCK

Preliminary studies have shown that dCK protein phosphorylation cannot be detected in leukemic cells incubated with [32P]-orthophosphate, probably due to low expression levels. To circumvent this problem, dCK was overexpressed in eukaryotic human embryonic kidney (HEK) 293T cells as a fusion protein with a 6xHistidine tag at the N-terminal position. The transfection was performed using jetPEI™ (PolyPlus Transfection, Illkirch, France). This method increased dCK activity by 500 to 1,000-fold. For in vivo labeling experiments, these HEK 293T cells were labeled with [32P]-orthophosphate and then lysed. Centrifuged lysates were purified using Clontech’s TALON Metal Affinity Resin (Cat. No. 635502). This one-step purification typically yielded a single 34 kDa band in Coomassie blue-stained gels (Figure 1), as expected for the 6xHistidine-tagged form of the 30.5 kDa dCK subunit (5). dCK was purified about 30-fold with a yield of approximately 20%. Autoradiography of the SDS-PAGE gel revealed a labeled 34 kDa band (Figure 2) corresponding to the band revealed by colloidal blue staining, as well as the band recognized by a monoclonal anti-poly(His) antibody. These results indicate that the labeled band is recombinant dCK and that it is constitutively phosphorylated.
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Figure 3. HPLC profile of $^{32}$P-labeled peaks after tryptic digestion of dCK from transfected HEK 293T cells. After incubation of HEK 293T cells with $^{32}$P-orthophosphate, radiolabeled dCK was purified using TALON® Resin, precipitated by methanol-chloroform, and subjected to SDS-PAGE. Bands corresponding to dCK were excised and digested with trypsin.

Labeled peptides were mixed with an unlabeled digest obtained in the same way and separated by reverse-phase narrow-bore HPLC in a linear acetonitrile gradient. Fractions were counted by Cerenkov radiation. Peaks containing peptides are labeled I–IV. Underlined peaks contain phosphorylated residues.

Identification of in vivo phosphorylation sites in dCK

Bands corresponding to dCK were excised from the gel and digested with trypsin. Peptides were separated by reverse-phase HPLC, and fractions were counted by Cerenkov radiation (Figure 3). Several radioactive peaks were detected, indicating the presence of multiple phosphorylation sites in the recombinant protein. Except for Peak I appearing in the flowthrough fraction, each radioactive peak was screened for phosphopeptide detection by neutral loss of $\text{H}_2\text{PO}_4^-$ (98 Da) by nano-ESI-MS/MS. This technique allowed the identification of four phosphorylated residues (Table I). In Peak IV, no phosphopeptide was detected. The relative labeling of the HPLC peaks was calculated in three separate experiments. Ser-74, representing 44.6 ± 6.2% of total $^{32}$P incorporation, appeared to be the major in vivo phosphorylation site. Moreover, site-directed mutagenesis experiments showed that phosphorylation of Ser-74 is crucial for dCK activity, whereas phosphorylation of the other identified sites did not appear to be essential. A more detailed description of this study is available (4).

Conclusion

$^{32}$P-labeled experiments demonstrated that dCK is constitutively phosphorylated after overexpression in eukaryotic cells and its activity depends on the phosphorylation state of Ser-74. Expression of dCK with a 6xHistidine tag enabled one-step purification with TALON Metal Affinity Resin, limiting loss of protein and reducing manipulation time, an added convenience when using $^{32}$P-labeled materials.

Table I: Sequences of dCK Phosphopeptides Determined by Nano-ESI-MS/MS$^1$

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences</th>
<th>Phosphorylated residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>$\text{pEF6/His}3\text{DPA, pTPPKR}$</td>
<td>Thr-3</td>
</tr>
<tr>
<td>III</td>
<td>$\text{ScpSFSApSSEGTR}_{\text{o}}$</td>
<td>Ser-11, Ser-15</td>
</tr>
<tr>
<td>IV</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>V</td>
<td>$\text{WeNVGSTQDEFELTM}_{\text{pSQK}}$</td>
<td>Ser-74</td>
</tr>
</tbody>
</table>

$^1$Labeled (His)$_6$ dCK HPLC peaks (Figure 3) were analyzed by nano-ESI-MS/MS. Phosphopeptides were identified by loss of 98 Da upon collision-induced dissociation, and the phosphorylated residue was further identified by fragmentation in MS$^3$ mode. The first two amino acids of peptide II, aspartate and proline, belong to the expression vector pEF6/His and are followed by Ala-2 of dCK; the initial methionine was absent because of the introduction of a BamHI restriction site in the dCK cDNA. c = acrylamide (propionamido)-modified cysteine or iodoacetamide (carboxamidomethyl)-modified cysteine. p = phosphorylated residue. M$_{ox}$ = oxidized Met. ND = not detected.

References