Characterization of Human UMP/CMP Kinase and Its Phosphorylation of 3′- and 1′-Form Deoxycytidine Analogue Monophosphates

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ABSTRACT

Pyrimidine nucleoside monophosphate kinase [UMP/CMP kinase (UMP/CMPK); EC 2.7.4.14] plays a crucial role in the formation of UDP, CDP, and dCDP, which are required for cellular nucleic acid synthesis. Several cytidine and deoxycytidine analogues are important anticancer and antiviral drugs. These drugs require stepwise phosphorylation to their triphosphate forms to exert their therapeutic effects. The role of UMP/CMPK for the phosphorylation of nucleoside analogues has been indicated. Thus, we cloned the human UMP/CMPK gene, expressed it in Escherichia coli, and purified it to homogeneity. Its kinetic properties were determined. UMP and CMP proved to be far better substrates than dCMP. UMP/CMPK used all of the nucleoside triphosphates as phosphate donors, with ATP and dATP being the best donors and CTP being the poorest. Furthermore, UMP/CMPK was able to phosphorylate all of the deoxycytidine analogue monophosphates that we tested. The relative efficiency was as follows: arabinofuranosyl-CMP > dCMP > β-l,2′,3′,5′-dideoxy-3′-thia-CMP > Gemcitabine monophosphate > β-l,2′,3′-dideoxy-CMP; β-l,2′,3′,5′-dideoxy-2′,3′-didehydro-5-fluoro-CMP; β-l,2′,3′,5′-dideoxy-5-fluoro-3′-thia-cytidine and β-l,2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine have been shown to be potent antihuman hepatitis B virus agents in vitro and in animal studies (18–22). In studies of other β-l,2′,3′-dideoxy-3′-deoxycytidine analogues, it was observed that l-β-D-arabinofuranosylcytidine and Gemcitabine. These agents need to be phosphorylated in a stepwise fashion by human kinases to their triphosphate forms to exert their activities. The first phosphorylation step is carried out by deoxycytidine kinase and uridine kinases (30). The pyrimidine nucleoside monophosphates are further phosphorylated by pyrimidine nucleoside monophosphate kinase, which is thought to be UMP/CMPK. The third step is carried out by nucleoside diphosphate kinases or other unidentified kinases.

Human and mammalian UMP/CMPs have been partially purified by a number of investigators, including our group (31–40). Some characterizations have been performed on these partially purified enzymes. Recently, a pig UMP/CMPK gene has been cloned and expressed in Escherichia coli, and the recombinant protein was purified to homogeneity (7). However, the enzyme activity and properties have not been well characterized, especially with regard to the phosphorylation of nucleoside analogue monophosphates. In this study, we cloned the cDNA of human UMP/CMPK, expressed it in E. coli, and purified the recombinant protein. The enzyme kinetics and phosphorylation of both natural nucleoside and nucleoside analogue monophosphates were studied, with special emphasis on the enzymatic conversion of several clinically important l-form nucleoside analogue monophosphates. The subcellular localization and protein form were determined using a specific antibody. Independently, van Rijmuyt et al. (41) have also cloned the human UMP/CMPK gene and have done some enzymatic characterization using the recombinant kinase. Detailed comparisons will be presented in the “Discussion” section.

INTRODUCTION

UMP/CMPK catalyzes the phosphoryl transfer from ATP to CMP, UMP, and dCMP, resulting in the formation of ADP and the corresponding nucleoside diphosphates (1). Both de novo and salvage pathways produce pyrimidine monophosphate, and these two pathways converge at the level of UMP/CMPK for the synthesis of pyrimidine diphosphate. Thus, UMP/CMPK plays an important role in pyrimidine synthesis. Protein sequences from different species show that the UMP/CMPK gene is highly conserved, suggesting an important role of this kinase in all of these organisms (2–9). Recently, a conditional lethal mutant isolated from Saccharomyces cerevisiae was described in which UMP/CMPK was mutated, indicating the essential role of the kinase in this organism (10). However, a detailed study of this enzyme has not been performed to date.

Deoxycytidine and cytidine analogues, such as 1-β-D-arabinofuranosylcytosine, 5-azacytidine, and 2′,2′-difluorodeoxycytidine (Gemcitabine), were shown to be useful for the treatment of patients with leukemia, lymphoma, or solid tumors (11). Deoxycytidine analogues, such as β-3′-dideoxycytidine and l-β-D-3′-dideoxycytidine (Gemcitabine), have been shown to have anti-HIV and antihuman hepatitis B virus activities (12–17). l-β-D-3′-dideoxycytidine was the first nucleoside analogue with an l-configuration to show therapeutic activity and, thus, defined a new category for the design of nucleoside analogues. β-l,2′,3′-dideoxy-5-fluoro-3′-thia-cytidine and β-l,2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine have been shown to be potent antihuman hepatitis B virus agents in vitro and in animal studies (18–22). In studies of other β-l,2′,3′-dideoxy-3′-deoxycytidine analogues, it was observed that l-β-D-arabinofuranosylcytidine is the only cytotoxic deoxycytidine analogue to act as a true chain terminator upon incorporation into DNA (23, 24). Preclinical and Phase I evaluations demonstrated its effectiveness against both leukemia and solid tumors (25–29). At present, l-β-D-arabinofuranosylcytidine is the only cytotoxic deoxycytidine analogue to act as a true chain terminator upon incorporation into DNA (23, 24). Preclinical and Phase I evaluations demonstrated its effectiveness against both leukemia and solid tumors (25–29). At present, l-β-D-arabinofuranosylcytidine and Gemcitabine. These agents need to be phosphorylated in a stepwise fashion by human kinases to their triphosphate forms to exert their activities. The first phosphorylation step is carried out by deoxycytidine kinase and uridine kinases (30). The pyrimidine nucleoside monophosphates are further phosphorylated by pyrimidine nucleoside monophosphate kinase, which is thought to be UMP/CMPK. The third step is carried out by nucleoside diphosphate kinases or other unidentified kinases.

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3 The abbreviations used are: UMP/CMPK, UMP/CMP kinase; l-β-2,3′,5′-dideoxy-3′-thia-CMP (3TC), β-l,2′,3′-dideoxy-3′-thiacytidine; aa, amino acid; l-β-D-3′-dideoxy-CMP, β-l,2′,3′-dideoxy-CMP; l-β-2,3′-dideoxy-5-fluoro-3′-thia-CMP, HPLC, high-performance liquid chromatography; AraCMP, 1-β-D-arabinofuranosylCMP, ORF, open reading frame; GST, glutathione-S-transferase; GFP, green fluorescent protein.

MATERIALS AND METHODS

Cloning of the Human UMP/CMPK Gene. Total RNA was extracted from KB cells (human epidermoid carcinoma). Five μg of total RNA were used in the reverse transcription reaction using SuperScript II (Life Technologies, Inc., Rockville, MD) and oligo(dT) primer. The reverse transcription product was subjected to PCR with the primers 5′-GTACGCTCCTCATGGTCGCCG-3′ and 5′-TGTCGCCAACATCTCAAGG-3′, which were designed according to the assembled EST sequences. Amplification was performed in a PTC-100 thermal controller (MJ Research, Watertown, MA). The amplified 1.2-kb product was cloned into the Eukaryotic TA Cloning Vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The
plasmid clones were checked by EcoRI cutting to contain the 1.2-kb fragment. Potential clones were confirmed by sequencing.

**Expression, Purification, and Thrombin Cutting of Recombinant UMP/CMPK.** Two sets of primers were used to amplify the desired fragments for the expression of 228-aa and 196-aa UMP/CMPK forms. The primers were designed to contain an NdeI site at the 5' end and a BamHI site at the 3' end for cloning purposes. The primer sets used were as follows: 5'-GGGAATTCCTATATGCT-GAGCGCCTGCGCGACG-3' and 5'-GGCCGATCCAGTACATGGCCCTC- TGGTCG-3' (for 228-aa UMP/CMPK); 5'-GGGAATTCCTATATGAGCGCGCTGTCGGTICT-3' and 5'-GGCCGATCCAGTACATGGCCCTC- TGGTCG-3' (for 196-aa UMP/CMPK). The amplified fragments were cut with NdeI and BamHI restriction enzymes and cloned into the PET-28a expression vector (Novagen, Madison, WI), which carries an NHEI-terminal His-tag/thrombin configuration. The 196-aa UMP/CMPK fragment was also cloned into vector pCR3.1 (Invitrogen) under the control of CMV promoter for eukaryotic expression. The clones containing the desired NdeI-BamHI fragments were selected and checked by sequencing. PET-28a-UMP/CMPK constructs were transformed into BL21-Gold(DE3)-competent E. coli cells (Stratagene, La Jolla, CA) for protein expression. The optimal expression conditions of recombinant UMP/CMPK were 0.5 mM isopropyl-1-thio-β-D-galactopyranoside induction for 2–3 h. The transformants were lysed in lysis buffer [40 mM Tris (pH 7.5), 10 mM NaCl, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, with or without 1 mM DTT] by sonication. The recombinant proteins were purified by Ni²⁺-NTA affinity chromatography (Invitrogen) and digested with biotinylated thrombin (Invitrogen) to remove the His-tag. The thrombin was removed by avidin-agarose beads according to the manufacturer’s instructions (Invitrogen). The proteins were passed through a Ni²⁺-column again to remove the His-tag. Using this procedure, we obtained enzymes that were usually >95% pure (as determined using silver staining). The enzyme was further purified on a FPLC chromatography (Mono Q column; Amersham Pharmacia Biotech, Piscataway, NJ).

**Western Blotting with Total Lysate of Human Cell Lines.** Human cell lines, although in different amounts (Fig. 2). The endogenous UMP/CMPK was detected in cell extracts using Western blotting. The kinase was expressed in all of the tested human cell lines, although in different amounts (Fig. 2). The putative ORF was predicted to encode a 228-aa protein with a predicted molecular mass of 26 kDa. Alignment of the UMP/CMPKs cloned from human and other species, however, indicated that the human enzyme might be a 196-aa protein (Fig. 1). We therefore constructed both 228-aa and 196-aa UMP/CMPK expression vectors. Both the 228-aa and the 196-aa forms of human UMP/CMPK were expressed as His-tagged fusion proteins to facilitate their purification from E. coli extracts. The proteins were purified to homogeneity; silver staining of SDS-PAGE revealed that they were >95% pure (data not shown). The His-tags were then removed by thrombin digestion. The enzyme properties of these two proteins were compared as shown below.

**Detection of Endogenous UMP/CMPK by a Specific Antibody.** To detect the expression of UMP/CMPK in human cell lines, rabbit antiserum was produced against recombinant protein (outlined in “Materials and Methods”). UMP/CMPK was detected in cell extracts by Western blotting. The kinase was expressed in all of the tested human cell lines, although in different amounts (Fig. 2). The endogenous UMP/CMPK appeared to be a single-size protein as shown by Western blotting of the human cell lines. The detected protein comigrated with the 196-aa recombinant UMP/CMPK proteins from E. coli and the transfectants of HeLa S3 cells. This result suggests that the 196-aa UMP/CMPK is the actual form of UMP/CMPK, and not the 228-aa form as suggested by van Rompay et al. (41).

**Subcellular Localization of UMP/CMPK.** Using the rabbit antiserum that we produced, we were able to determine subcellular localization of UMP/CMPK by indirect immunofluorescent confocal microscopy. UMP/CMPK protein was predominantly localized to the cytoplasm of HeLa S3 (Fig. 3) and the other cell lines (data not shown). Some signal was detected in the nucleus (<15%), but none was detected in the mitochondria. These results were further confirmed by subcellular fractionation, Western blotting, and activity assays (data not shown). We detected no UMP/CMPK protein or specific binding of antibodies. UMP/CMPK protein was targeted first by rabbit polyclonal anti-UMP/CMPK antibody (1:200) and subsequently by FITC-conjugated antirabbit IgG (1:100; Sigma). In the control experiment, purified UMP/CMPK protein was added to compete for binding with anti-UMP/CMPK antibody in the primary hybridization step. Nuclei were counterstained with propidium iodide (50 ng/ml). Cells were sealed in antiadherent reagent (Molecular Probes, Eugene, OR). Confocal micrographs were scanned by a laser scan confocal microscope (LSM 510; Zeiss).

**RESULTS**

**Cloning and Expression of the Human UMP/CMPK Gene in E. coli.** To clone the human UMP/CMPK gene, we retrieved the pig UMP/CMPK DNA sequence from GenBank. The pig UMP/CMPK DNA sequence (7) was used to search the human EST database. Several homologous sequences were found, retrieved from the data bank, and aligned using the GCG Pileup program. A 1.3-kb sequence was assembled, from which a single ORF highly homologous to the pig UMP/CMPK coding sequence was derived. According to the assembled sequence, one set of primers covering a 1.2-kb DNA sequence was designed. A single DNA product was amplified by PCR from human cell cDNA. The DNA fragment was cloned and sequenced (data not shown). The DNA sequence was identical to the sequence published by van Rompay et al. (41). The putative ORF was predicted to encode a 228-aa protein with a predicted molecular mass of 26 kDa. Alignment of the UMP/CMPKs cloned from human and other species, however, indicated that the human enzyme might be a 196-aa protein (Fig. 1). We therefore constructed both 228-aa and 196-aa UMP/CMPK expression vectors. Both the 228-aa and the 196-aa forms of human UMP/CMPK were expressed as His-tagged fusion proteins to facilitate their purification from E. coli extracts. The proteins were purified to homogeneity; silver staining of SDS-PAGE revealed that they were >95% pure (data not shown). The His-tags were then removed by thrombin digestion. The enzyme properties of these two proteins were compared as shown below.

**Antibody Production and Western Blotting.** Serum was taken and tested for specific antibody production 2 weeks after the infection. The antibodies were affinity purified from the serum that we produced, and then these two proteins were compared as shown below.

**Detection of Endogenous UMP/CMPK by a Specific Antibody.** To detect the expression of UMP/CMPK in human cell lines, rabbit antiserum was produced against recombinant protein (outlined in “Materials and Methods”). UMP/CMPK was detected in cell extracts by Western blotting. The kinase was expressed in all of the tested human cell lines, although in different amounts (Fig. 2). The endogenous UMP/CMPK appeared to be a single-size protein as shown by Western blotting of the human cell lines. The detected protein comigrated with the 196-aa recombinant UMP/CMPK proteins from E. coli and the transfectants of HeLa S3 cells. This result suggests that the 196-aa UMP/CMPK is the actual form of UMP/CMPK, and not the 228-aa form as suggested by van Rompay et al. (41).
activity in the purified mitochondrial extract. We also overexpressed the UMP/CMPK constitutively in HeLa S3 cells (3-fold overexpression as determined by Western blotting and activity assay). We detected more protein in both the nucleus and the cytosol; however, the overexpression of the kinase resulted in a higher UMP/CMPK protein ratio of nucleus to cytosol. The immunofluorescence was competed out with purified recombinant UMP/CMP protein in A and B to a different extent. The lighter staining, after competition, in the cytosolic compartment in B was either nonspecific or simply could not be competed by recombinant UMP/CMP protein because of a different conformation.

Enzymatic Properties of the Human Recombinant UMP/CMPK. Both the 228-aa and 196-aa recombinant enzymes were characterized in terms of their substrate specificity for natural substrates, UMP, CMP, and dCMP. The two enzymes exhibited identical properties with respect to all three substrates, both in terms of $K_m$ and relative activity (Table 1). The $K_m$s for CMP and UMP were similar but much lower than that for dCMP. In addition, the relative activities for UMP and CMP were much higher than that for dCMP. These results suggest that dCMP is a poor substrate for this enzyme compared with CMP and UMP. The $K_m$ for each substrate was shown to be different. The $K_m$ for dCMP (25 $\mu$M) was lower than that of UMP (204 $\mu$M) or CMP (211 $\mu$M; Table 1). Because these two enzymes behaved almost identically and we were able to detect only the 196-aa UMP/CMPK form in the cell lines under study, we focused our further enzymatic studies on the 196-aa recombinant enzyme.

It has been shown that the activity of the partially purified UMP/CMPK could be activated by reducing agents such as DTT. Thus, we studied the effect of reducing agents on the human recombinant UMP/CMPK in more detail. As shown in Fig. 4, the recombinant enzyme was almost inactive, or showed <10% activity in the absence of reducing agents, and its activity could be activated by DTT, 2-mercaptoethanol, and reduced thioredoxin in a dose-dependent manner. Because thioredoxin is an important reducing agent in vivo...
and the concentrations we used were in the physiological range, these results suggest that UMP/CMPK activity could be regulated by redox potentials within cells.

We next tested the phosphate donor specificities of UMP/CMPK, using UMP, CMP, and dCMP as phosphate acceptors. As shown in Table 2, human UMP/CMPK uses a broad spectrum of nucleoside triphosphates as phosphate donors. ATP and dATP were the best phosphate donors, whereas CTP was the poorest. dGTP and TTP were efficient phosphate donors for UMP and dCMP, but not for CMP. GTP, UTP, and dCTP were moderately efficient phosphate donors for all three phosphate acceptors.

There is a strong feedback inhibition for deoxycytidine kinase by dCTP (45, 48). We investigated the possible feedback inhibition of UMP/CMPK by pyrimidine nucleoside triphosphates. As shown in Table 3, CTP inhibited UMP phosphorylation by 80% and CMP and dCMP phosphorylation by 60%, with less inhibition by UTP and dCTP on UMP phosphorylation. No significant inhibition by TTP on UMP, CMP, and dCMP phosphorylation was observed. The responses of UMP phosphorylation in the presence of CTP, UTP, and dCTP were different from those of CMP and dCMP phosphorylation. We also examined whether CTP could alter the $K_m$ of dCMP in the presence of MgATP. No change in $K_m$ was observed.

![Image](Fig. 3. Detection of intracellular UMP/CMPK of HeLa S3 cells by immunofluorescent microscopy. HeLa S3-pCR3.1 (A) and HeLa S3-pCR3.1UMP/CMPK (B) cells were fixed, immunofluorescently stained with antibodies, and counterstained with propidium iodide as described in “Materials and Methods.” Green indicates UMP/CMPK; red indicates propidium iodide staining. Top panels in A and B were probed with anti-UMP/CMPK antisera without recombinant UMP/CMPK protein competition. Bottom panels in A and B were probed with anti-UMP/CMPK antisera with recombinant UMP/CMPK protein competition.)
Phosphorylation of Pyrimidine and Deoxycytidine Analogue Monophosphates. Several deoxycytidine analogues are important anticancer or antiviral agents. For this reason, whether the monophosphate derivatives of these agents can be phosphorylated by recombinant UMP/CMPK may have clinical relevance. As shown in Table 4, all of the compounds tested (structures shown in Fig. 5) were appropriate substrates for the human UMP/CMPK. AraCMP was a better substrate for this kinase than dCMP (5-fold). Gemcitabine monophosphate and L(-)-SddCMP were equally as good substrates as dCMP. D-ddCMP, L-ddCMP, L(-)-FSddCMP and L-dioxolane-CMP were all poor substrates with a relative efficiency of 20% of that of dCMP. L-dioxolane-CMP was the poorest substrate of all of the compounds tested. Importantly, this enzyme was capable of phosphorylating both D- and L-form deoxycytidine analogue monophosphates. Except for L(-)-SddCMP, all of

Table 2 Phosphate donor specificity of the recombinant UMP/CMPK
The phosphate donor and acceptor concentrations were 1 mM. The reaction was performed in the presence of 8 mM Mg\(^2+\). Values are presented as the percentage of ATP sample (mean ± SD from three independent experiments).

<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>Phosphate acceptor (% of ATP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UMP</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>CTP</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>UTP</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>dATP</td>
<td>115 ± 11</td>
</tr>
<tr>
<td>dGTP</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>dCTP</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>TTP</td>
<td>74 ± 5</td>
</tr>
</tbody>
</table>

Table 3 Inhibition of the recombinant UMP/CMPK by pyrimidine nucleoside triphosphates
The reactions were performed in the presence of 1 mM ATP and 8 mM Mg\(^2+\). The additive and phosphate acceptor concentrations were 1 mM. Values are presented as the percentage of ATP-only samples (mean ± SD from three independent experiments).

<table>
<thead>
<tr>
<th>Additive</th>
<th>Phosphate acceptor (% of ATP-only)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UMP</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>CTP</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>UTP</td>
<td>63 ± 11</td>
</tr>
<tr>
<td>dCTP</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>TTP</td>
<td>94 ± 9</td>
</tr>
</tbody>
</table>

Phosphorylation of Pyrimidine and Deoxycytidine Analogue Monophosphates. Several deoxycytidine analogues are important anticancer or antiviral agents. For this reason, whether the monophosphate derivatives of these agents can be phosphorylated by recombinant UMP/CMPK may have clinical relevance. As shown in Table 4, all of the compounds tested (structures shown in Fig. 5) were appropriate substrates for the human UMP/CMPK. AraCMP was a better substrate for this kinase than dCMP (5-fold). Gemcitabine monophosphate and L(-)-SddCMP were equally as good substrates as dCMP. D-ddCMP, L-ddCMP, L(-)-FSddCMP and β-L-2',3'-dideoxy-2',3'-didehydro-5-fluoro-CMP were all poor substrates with a relative efficiency of ~20% of that of dCMP. β-L-dioxolane-CMP was the poorest substrate of all of the compounds tested. Importantly, this enzyme was capable of phosphorylating both D- and L-form deoxycytidine analogue monophosphates. Except for L(-)-SddCMP, all of
Table 4 Phosphorylation of pyrimidine and deoxycytidine analogue monophosphates by human UMP/CMPK

The reactions were performed at 37°C in the presence of 1 nmol MgATP and 750 μM phosphate acceptor. Calculations for relative V_{max}, K_{m}, and relative efficiency are as mentioned in Table 1. Activity for dCMP phosphorylation was 33 ± 4 μmol/mg/min. Values are presented as mean ± SD from three independent experiments.

<table>
<thead>
<tr>
<th>Phosphate acceptor</th>
<th>Relative V_{max} (%)</th>
<th>K_{m} (μM)</th>
<th>Relative efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCMP</td>
<td>100</td>
<td>500 ± 54</td>
<td>100</td>
</tr>
<tr>
<td>AraCMP</td>
<td>827 ± 19</td>
<td>917 ± 83</td>
<td>451</td>
</tr>
<tr>
<td>Gemcitabine monophosphate</td>
<td>94 ± 10</td>
<td>581 ± 25</td>
<td>81</td>
</tr>
<tr>
<td>d-ddCMP</td>
<td>10 ± 2</td>
<td>272 ± 53</td>
<td>18</td>
</tr>
<tr>
<td>L-(-)-OddCMP*</td>
<td>19 ± 3</td>
<td>1037 ± 52</td>
<td>9</td>
</tr>
<tr>
<td>L-(-)-SddCMP</td>
<td>92 ± 10</td>
<td>494 ± 94</td>
<td>93</td>
</tr>
<tr>
<td>L-(-)-FSDdCMP</td>
<td>9 ± 2</td>
<td>250 ± 62</td>
<td>18</td>
</tr>
<tr>
<td>L-(-)-Fd4CMP</td>
<td>9 ± 2</td>
<td>228 ± 68</td>
<td>19</td>
</tr>
</tbody>
</table>

* L-(-)-OddCMP, β-L-dioxolane-CMP; L-(-)-Fd4CMP, β-L-2‘,3’-dideoxy-2‘,3’-didehydro-5-fluoro-CMP.

The dideoxythiouridine analogue monophosphates were poor substrates, irrespective of d or L configuration.

**DISCUSSION**

Our laboratory is interested in the enzymes involved in the activation of nucleoside analogues. In this study we focused on the enzyme that phosphorylates deoxythiouridine analogue monophosphates to their respective diphosphate forms. We cloned the human UMP/CMPK gene and characterized its activities in detail. Independently, van Rompay et al. (41) cloned the UMP/CMPK gene, using a similar strategy. In their report, they expressed the 228-aa UMP/CMPK as a fusion protein to GST and characterized the enzymatic properties of this recombinant protein. Using UMP/CMPK fused to GFP, they were able to show that this enzyme was localized to both the nucleus and the cytosol of UMP/CMPK-transfected cells. The fluorescence was distributed homogeneously in the cells.

To detect endogenous UMP/CMPK protein in human cell lines, we developed a specific antibody against it. The human UMP/CMPK protein is localized predominantly to the cytosol of human cells as detected by indirect immunofluorescent confocal microscopy. This result correlates with the subcellular fractionation data of previous reports (47), including our own. The distribution pattern of UMP/CMPK-overexpressed transfectants is different from that of nontransfected cells. The distribution is homogeneous in the UMP/CMPK-overexpressed cells, as reported by van Rompay et al. (41). We could not detect UMP/CMPK protein or activity in purified mitochondrial extracts. A mitochondrial dCTP import system was recently identified by our laboratory (48), which may explain the absence of detectable UMP/CMPK levels in the mitochondria.

There are two putative translational start codons near the 5’ end of the UMP/CMPK cDNA in the same ORF, one encoding a 228-aa protein and the other encoding a 196-aa protein (Fig. 1). According to the previously published NH2-terminal peptide sequences of UMP/CMPK from pig (7), the second methionine might be the translational start codon. The UMP/CMPKs from different human cell lysates were detected by Western blotting as proteins with the same size as the recombinant 196-aa protein expressed in E. coli or in HeLa S3 transfectants. This result indicates that the 196-aa form is the actual form of UMP/CMPK in the cells. We could not, however, rule out the existence of 228-aa protein in cell lines that we did not examine or the expression of trace amounts of 228-aa protein that we were unable to detect.

Our purified recombinant proteins contained only three extra amino acid residues at their NH2 termini after thrombin cutting and more closely resembled the native enzyme. We then investigated the recombinant enzymes in detail with regard to the natural substrates and clinically important anticancer and antiviral pyrimidine nucleoside analogues, especially the L-configuration analogues. Our data showed that the 228-aa and 196-aa UMP/CMPKs behaved almost the same with regard to the phosphorylation of natural substrates (Table 1). UMP and CMP were the best, and dCMP was a fairly good substrate, whereas dUMP was poorly phosphorylated (relative rate <20% of dCMP phosphorylation). These results are similar to those for partially purified UMP/CMPKs from mammalian cells (31–40). The K_{m} for UMP and CMP of the recombinant enzymes were much lower than those reported by van Rompay et al. (15 and 67 μM versus 500 and 1600 μM, respectively). Our data were, however, similar to those obtained in several studies in which partially purified enzymes were used (1, 31, 32, 33, 35) and more likely to fit physiological concentrations (49). The specific activities we measured for UMP, CMP, and dCMP phosphorylation were ~300, 350, and 30 μmol/mg/min, re-

Fig. 5. Cytidine and deoxycytidine analogues used in this study. ddC, β-d-2‘,3’-dideoxythiouridine; L-(-)-ddC, β-L-2‘,3’-dideoxythiouridine; L-(-)-FsddC, β-L-2‘,3’-dideoxy-5-fluoro-2‘,3’-didehydro-5-fluoro-L-thiacytidine; L-(-)-Fd4C, β-L-2‘,3’-dideoxy-2‘,3’-didehydro-5-fluoro-L-thiacytidine.
spectively (Fig. 3; Tables 1 and 2). These activities were also much higher than the V_{max} reported by van Rompay et al. (41). The considerable differences between our and other group’s results may be attributable to different assay methods or to the fact that the protein they used was fused to the large GST protein.

UMP/CMPK can use a broad spectrum of phosphate donors but the best phosphate donors are ATP and dATP, as was determined for partially purified mammalian UMP/CMPK (31, 36). ATP should be the actual phosphate donor because the dATP concentration is low in the cells. Interestingly, the K_{m} for dCMP was much lower than those for UMP and CMP. There is no inhibition of dCMP phosphorylation by CMP or CTP (1 mM), but the inhibition was not obvious at the lower concentration of CTP (1 mM; 50% inhibition). Therefore, the significance of the inhibition needs to be further explored. No significant inhibition by TTP (1 mM) was observed. This seems reasonable because dUMP and TMP are poor substrates for UMP/CMPK. The results shown for TTP in Table 3 and for ATP, dATP, and GTP (the relative activities are more than or near 100%) demonstrate that 8 mM Mg^{2+} combined with 1 mM ATP, 1 mM nucleotide triphosphate, and 1 mM nucleoside monophosphate was not inhibitory.

According to the results we obtained with the recombinant enzyme, it might be difficult for dCMP to be phosphorylated efficiently in vivo because the physiological concentrations of UMP and CMP are higher than that of dCMP. We explored this question further. We found that the relative activity of dCMP phosphorylation by cell lysates was much higher than that of the recombinant enzyme (data not shown). K_{m} for dCMP phosphorylation by cell lysates were the same as that for recombinant enzyme. Several possibilities could explain these findings: posttranslational modification or interaction with other protein(s), increasing dCMP phosphorylation by UMP/CMPK, or the presence of another unidentified enzyme with dCMP-phosphorylating activity. We searched for possible protein modification sites, using the Prosite program, and found several putative kinase phosphorylation sites in the UMP/CMPK protein sequence. No other significant consensus sites were found. We were able to demonstrate that protein kinase A, but not casein kinase II and protein kinase C, could phosphorylate the UMP/CMPK protein in vitro (data not shown). However, the relative activities of UMP, CMP, and dCMP phosphorylation were not changed by protein kinase A phosphorylation at either low (50 and 100 μM) or high (1000 μM) concentrations of these substrates (data not shown). We are now using the yeast two-hybrid system to identify associated proteins that could change the relative activity of UMP/CMPK. The possibility of another, yet unidentified, dCMP kinase is also being further investigated.

It is known that reducing agents can activate partially purified UMP/CMPK. Here we confirmed this observation with purified human recombinant enzyme and showed that the activation could be dramatic (>50-fold). In the report by van Rompay et al. (41), the DTT treatment was not good for UMP, CMP, and dUMP phosphorylation. This may be a result of the fusion with GST protein. It is noticeable that the dose and time required to activate the kinase activity were different for UMP, CMP, and dCMP. The observation that physiological concentrations of thioredoxin can fully activate kinase activity strongly suggests that UMP/CMPK could be regulated by the redox potential in cells. This possibility is being explored at present. Interestingly, the recombinant UMP/CMPK from Entamoeba histolytica does not require activation by DTT and 2-mecaptoethanol (8). Protein sequence analysis showed that the UMP/CMPKs from humans, pigs, and mice shared all four cysteine residues, which were not found in the UMP/CMPK from E. histolytica. This observation suggests that the redox regulation may be developed later during evolution.

The most important information from our studies is that all of the monophosphates of d- and l-configuration deoxycytidine analogues can be phosphorylated to the corresponding diphosphate form by this recombinant UMP/CMPK. The structure and function relationships are summarized below. Both 2'-OH and 3'-OH are important for phosphorylation, whereas 3'-OH is the most important determinant for activity (CMP and UMP are much better substrates than dCMP and dUMP). The enzyme shows some selectivity for the ribose configuration, and the cis form appears to be more favorable (CMP versus AraCMP). It shows no obvious difference with regard to stereospecificity, as evidenced by the comparison of d- and l-ddCMP. The comparisons of l-(-)-SddCMP with l-(-)-FsddCMP and dUMP with TMP (relative rates <20% and 0.1% of dCMP phosphorylation, respectively) suggest that any group at the 5 position of the base could have a significant impact on the activity. The enzyme favors a “sulfur” over an “oxygen” at the 2’ position of the ribose ring [l-(-)-SddC versus l-(-)-OddC].

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