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CLONING AND EXPRESSION OF HUMAN URIDINE PHOSPHORYLASE*

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Using a mouse cDNA probe we have identified a human uridine phosphorylase cDNA clone from the cDNA library of a human colorectal tumor cell line, HCT116. The recombinant human uridine phosphorylase expressed in COS-7 cells demonstrated specific enzyme activity with uridine as the substrate; this activity was inhibited by the competitive inhibitor 2,2'-anhydro-5-ethyluridine. Northern blot analysis with the cDNA as a probe demonstrated high levels of mRNA expression in several tumor cell lines but very low level in normal cell, WI-38. The expression of uridine phosphorylase mRNA in HCT-116 cells was further enhanced by treating the cells with vitamin D3 and the inflammatory cytokines: tumor necrosis factor α, interleukin 1α and interferon γ.

The two known kinds of pyrimidine nucleoside phosphorylases, uridine phosphorylase (UdRPase; EC 2.4.2.3) and thymidine phosphorylase (TdRPase; EC 2.4.2.4), in the presence of orthophosphate, catalyze the reversible phosphorolysis of uridine and thymidine or deoxyuridine, respectively, to free bases and ribose-1-phosphate or deoxyribose-1-phosphate. Pyrimidine nucleoside phosphorylases can add ribose or deoxyribose to pyrimidine bases to form nucleosides that can be

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incorporated into RNA or DNA. Human TdRPase has been purified, cloned and shown to be identical to platelet-derived endothelial cell growth factor (PD-ECGF;1-3) and gliostatin (4), but human UdRPase has not been identified yet.

Recently we purified the murine UdRPase protein from a mouse cancer cell line, Colon-26, and cloned the cDNA (5). Using the mouse UdRPase probe, we cloned human UdRPase cDNA from a human colon cancer cell line, HCT116. We have tested the expression of the UdRPase gene in various human cancer cell lines and have found that upregulation occurs in HCT116 cells treated with cytokines and vitamin D3.

MATERIALS AND METHODS

Cell Lines-- The followimg 23 cell lines were cultured in optimal medium containing 10% fetal calf serum: normal fibroblast cell line (WI-38), gastric cancer cell lines (MKN1, MKN28, MKN45, AGS and KATO III), colon cancer cell lines (WiDr, HCT116, Lovo, DLD1, Colo201, Colo205, Colo320DM and CXF280), breast cancer cell lines (MCF-7, ZR-75-1 and MDA-MB-231), bladder cancer cell line (T-24), lung cancer cell line (A549), myeloblastic cell line (HL60), monocytoid cell line (U937), hepatoblastoma cell line (HepG2) and cervix cancer cell line (HeLa). HCT116 cells were separately treated with 1-α-25-dihydroxyvitamin D3 (final 200nM), human TNFα (final 1,000 units/ml), human IL-1α (final 100 units/ml), human IFNγ (final 10units/ml) (all obtained from Hoffman-La-Roche, Basle, Switzerland). They were also treated with a combination of the above concentrations of the three kinds of cytokines. All cell lines were cultured for 24h in a humidified, 5% CO_2 atmosphere at 37°C.

Library Construction and Cloning-- Preparation of the mRNA from HCT116 cells, cDNA synthesis and ligation to λgt11 were done as described previously (5,6). $5x10^5$ plaques were screened by the ^{32}P-labeled 1.3kb of mouse UdRPase cDNA probe. We obtained two independent clones having insert sizes of approximately 1.4 kb and 0.95 kb. These 1.4 kb and 0.95 kb cDNAs were subcloned into the mammalian expression vector pSG5 (7), pUP1-SG5 (1.4kb) and pUP2-SG5 (0.95kb).

cDNA Expression in COS-7 Cells and UdRPase assay-- 20 μg each of pSG5, pUP1-SG5 and pUP2-SG5 were transfected into COS-7 cells by LipofectAMINE (GIBCO BRL). After the cells were incubated for 24 h, they were harvested and sonicated. The cell extracts were dialyzed against the reaction buffer and UdRPase was

assayed in the reaction mixture containing 50mM potassium phosphate buffer (pH 7.4) and 10 mM uridine or thymidine. UdRPase inhibitor, 2,2'-anhydro-5-ethyluridine (100 μM) was added to the reaction mixture to test the substrate specificity. The enzyme reaction was performed at 37°C for 30 min and terminated by adding methanol. After removal of the precipitate by centrifugation, the supernatant was applied to a high-performance liquid chromatography column (6x200mm) of ERC-ODS-1171 (ERMA CR. Inc.). The amounts of uracil and thymine produced by phosphorolysis from uridine and thymidine were measured with a UV detector (265nm). The protein concentration was determined by the method of Lowry et. al. (8).

Northern Blotting and Hybridization-- Total RNA was extracted from log-phase cultures of the 23 cell lines, as well as from the vitamin D3 and cytokine- treated HCT116 cells. Then, 20 μg of the total RNAs were separated by electrophoresis on 1% agarose gels and transferred onto nylon membranes (Hybond N; Amersham-Buchler, Braunsschweig, Germany). ^{32}P- labeled UdRPase cDNA and β-actin cDNA (control probe) were hybridized to the membranes.

RESULTS

Identification and Expression of UdRPase-- Two different sizes of cDNA were obtained from the HCT116 cDNA library; the longer one is 1349 bp, the shorter one, 965 bp. The complete sequence of the longer cDNA is shown in Fig.1; the underlined 392 nucleotides are spliced out from the longer cDNA to leave the shorter one. The sequences of human, murine and *E. coli* UdRPase proteins are compared in Fig. 2. The deduced amino acid sequence of the human UdRPase cDNA showed 78.5 % identity with that of the mouse UdRPase. The deduced amino acids of the shorter cDNA numbered only 36, because the frame was shifted.

The enzyme activity was examined by transfecting the cDNA into COS-7 cells and assaying the cell lysates with uridine. The longer cDNA- transfectant showed the uridine phosphorylase activity (Table 1) but the shorter cDNA- transfectant did not (data not shown). The substrate specificity of the UdRPase cDNA- transfectant was tested by two different substrates, uridine and thymidine. As summarized in Table 1, the specific activity of UdRPase in cells transfected with pUP1-SG5 was 54-fold higher than that in cells transfected with the vector alone when uridine was used as the substrate. There was little activity detected with thymidine. The enzymatic

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ATTCGAGTGCTCCGGAGAACAGACCCGCGCCCCGCCGTCCGCGAGCCTCCCGAGAGCCGT   60
CCCTTCGTCCGGCCCTGGAGCATTGCGTTTGTCGCCGGTGTCGCAGTGCGAGGATGGCGC  120
CGCGGGTGTAGCGGCTCTCTGCGCAGGCCGAGTGGGCCCAGAGAAGCGAGGAACTCCCCA  180
GATCGCCGACACGTCTCGTCTCCTGTCCCAATTCAGGGCTTGGTGAGGTGACTCGCGGTC  240
GCGGGTGACTCGCCGGCAGGACACTGCCTGGAACGCCTGGAGCGCCTCCCACTGCAGACG  300

TCTGTCCGCCTCCAGCCGCTCTCCTCTGACGGGTCCTGCCTCAGTTGGCGGAATGGCGGC  360
                                                  M  A  A

CACGGGAGCCAATGCAGAGAAAGCTGAAAGTCACAATGATTGCCCCGTCAGACTTTTAAA  420
 T  G  A  N  A  E  K  A  E  S  H  N  D  C  P  V  R  L  L  N

TCCAAACATAGCAAAAATGAAAGAAGATATTCTCTATCATTTCAATCTCACCACTAGCAG  480
 P  N  I  A  K  M  K  E  D  I  L  Y  H  F  N  L  T  T  S  R

ACACAATTTCCCAGCCTTGTTTGGAGATGTGAAGTTTGTGTGTGTTGGTGGAAGCCCCTC  540
 H  N  F  P  A  L  F  G  D  V  K  F  V  C  V  G  G  S  P  S

CCGGATGAAAGCCTTCATCAGGTGCGTTGGTGCAGAGCTGGGCCTTGACTGCCCAGGTAG  600
 R  M  K  A  F  I  R  C  V  G  A  E  L  G  L  D  C  P  G  R

AGACTATCCCAACATCTGTGCGGGAACTGACCGCTATGCCATGTATAAAGTAGGACCGGT  660
 D  Y  P  N  I  C  A  G  T  D  R  Y  A  M  Y  K  V  G  P  V

GCTGTCTGTCAGTCATGGTATGGGCATTCCTTCTATCTCAATCATGTTGCATGAGCTCAT  720
 L  S  V  S  H  G  M  G  I  P  S  I  S  I  M  L  H  E  L  I

AAAGCTGCTGTACTATGCCCGGTGCTCCAACGTCACTATCATCCGCATTGGCACTTCTGG  780
 K  L  L  Y  Y  A  R  C  S  N  V  T  I  I  R  I  G  T  S  G

TGGGATAGGTCTGGAGCCCGGCACTGTGGTCATAACAGAGCAGGCAGTGGATACCTGCTT  840
 G  I  G  L  E  P  G  T  V  V  I  T  E  Q  A  V  D  T  C  F

CAAGGCAGAGTTTGAGCAGATTGTCCTGGGGAAGCGGGTCATCCGGAAAACGGACCTTAA  900
 K  A  E  F  E  Q  I  V  L  G  K  R  V  I  R  K  T  D  L  N

CAAGAAGCTGGTGCAGGAGCTGTTGCTGTGTTCTGCAGAGCTGAGCGAGTTCACCACAGT  960
 K  K  L  V  Q  E  L  L  L  C  S  A  E  L  S  E  F  T  T  V

GGTGGGGAACACCATGTGCACCTTGGACTTCTATGAAGGGCAAGGCCGTCTGGATGGGGC 1020
 V  G  N  T  M  C  T  L  D  F  Y  E  G  Q  G  R  L  D  G  A

TCTCTGCTCCTACACGGAGAAGGACAAGCAGGCGTATCTGGAGGCAGCCTATGCAGCCGG 1080
 L  C  S  Y  T  E  K  D  K  Q  A  Y  L  E  A  A  Y  A  A  G

CGTCCGCAATATCGAGATGGAGTCCTCGGTGTTTGCCGCCATGTGCAGCGCCTGCGGCCT 1140
 V  R  N  I  E  M  E  S  S  V  F  A  A  M  C  S  A  C  G  L

CCAAGCGGCCGTGGTGTGTGTCACCCTCCTGAACCGCCTGGAAGGGGACCAGATCAGCAG 1200
 Q  A  A  V  V  C  V  T  L  L  N  R  L  E  G  D  Q  I  S  S

CCCTCGCAATGTGCTCAGCGAGTACCAGCAGAGGCCGCAGCGGCTGGTGAGCTACTTCAT 1260
 P  R  N  V  L  S  E  Y  Q  Q  R  P  Q  R  L  V  S  Y  F  I

CAAGAAGAAACTGAGCAAGGCCTGAGCGCTGCCCTGCACCTCCGCAGACCTGCTGTGATG 1320
 K  K  K  L  S  K  A  *

ACTTGCCATTAAAAGCATTGTCCAAACCC                                  1349
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Fig.1. **Nucleotide and deduced amino acid sequence of human uridine phosphorylase.** The deduced amino acid sequence is shown under the nucleotide sequence. The underline indicates the region of the nucleotide sequence deleted to form the shorter cDNA.

```
S----------------------------KSDV---FHLGLTKNDLQG-
MAATGTEAKDLENHHNDCFIQLSNPNIAAMKEDVLYHFNLSTSTHDFPAM
MAATGANAEKAESHN-DCPVRLLNPNIAKMKEDILYHFNLTTSRHNFPAL
                                * *.   *.*  . ... .

---ATLAIVPGDPDRVEKIAALMDKPVKLASH-REF------TTWRAELD
FGDVKFVCVGGSSSRMNTFIKYVAAELGLDHPGKEYPNICAGTDRYAMYK
FGDVKFVCVGGSPSRMKAFIRCVGAELGLDCPGRDYPNICAGTDRYAMYK
     .  * * . *.  .   .   . *    ...      * . *

GKPVIVCSTGIGGPSTSIAVEELAQL------GIRTFLRIGTTGAIQPHI
AGPVLSVSHGMGIPSIGIMLHELIKMLYHARCSNITIIRIGTSGGIG--L
VGPVLSVSHGMGIPSISIMLHELIKLLYYARCSNVTIIRIGTSGGIG--L
  **.  * *.* ** .* ..** ..      .  *..****.*.*   .

NVGDVLVTTASVR----------LDGASLHFAPLEFPAVADF-ECTTAL
EPGSVVITQQAVNECFKPEFEQIVLGKRVIRNTNLDAQLVQELVQCSSDL
EPGTVVITEQAVDTCFKAEFEQIVLGKRVIRKTDLNKKLVQELLLCSAEL
. * *..*  .*            *.   .. . *.   * ..  *.. *

VEAAKSIGATTHVGVTASSDTFYPGQERYD----TYSGRVVRHFKGSMEE
NEFPMVVGNTM------CTLDFYEGQGRLDGALCSYTEKDKQSY---LRA
SEFTTVVGNTM------CTLDFYEGQGRLDGALCSYTEKDKQAY---LEA
 *    .* *        .  ** ** * *    .*. .  . .   .

WQAMGVMNYEMESATLLTMCASQGLRAGMVAGVIVNRTQQEIPNA--ETM
AHAAGVRNIEMESSVFATMCGACGLKAAVVCVTLLDRLQGDQINTPHDVL
AYAAGVRNIEMESSVFAAMCSACGLQAAVVCVTLLNRLEGDQISSPRNVL
  * ** * ****. . .**.. **.*..*   ...* . .  ..  . .

KQTESHAVKIVVEAARRLL---
VEYQQRPQRLVGHFIKKSLGRA
SEYQQRPQRLVSYFIKKKLSKA
 . . .. ..*    .. *
```

Fig.2. **Alignment of the amino acid sequences of the *E.coli*, mouse and human uridine phosphorylase with clustal program.** The *asterisks* and *periods* under the amino acid sequence indicate identical amino acids and conservative amino acid substitutuions,respectively.

Table1. Specific activities of recombinant human UdRPase in extracts of transfected COS-7 cells

Transfected gene	Substrate	
	Uridine*	Thymidine*
Human UdRPase-pSG5	300 ± 36	1.5 ± 0.34
pSG5 vector alone	5.6 ± 1.0	1.3 ± 0.62
% inhibition by 2,2'-anhydro-5-ethyluridine	88	N.D.

*10^3units/mg protein, where 1unit = 1μmol/min uracil or thymine converted from uridine or thymidine.
N.D. ; Not Done

activity of UdRPase was almost completely inhibited by 100 μM 2,2'-anhydro-5-ethyluridine.

UdRPase Expression in Human Tumor Cell Lines and the Induction by Cytokines and Vitamin D3-- Northern blot analysis was used to examine the expression of UdRPase mRNA in 22 human tumor cell lines and one normal fibroblast cell line, WI-38. The RNA was detected in 13 of the 22 tumor lines, and little RNA was detected in WI-38. Expression of UdRPase was increased by treatment with vitamin D3 and the cytokines, TNFα, IL-1α and IFNγ (Fig. 2).

DISCUSSION

Although there are two kinds of pyrimidine nucleoside phosphorylases in humans, TdRPase and UdRPase, only TdRPase has been identified as a platelet-derived endothelial cell growth factor, and human UdRPase has not been identified yet. To study the function of human UdRPase, we tried to identify human UdRPase. To begin with, we identified mouse UdRPase, because UdRPase levels are much higher in the mouse tumor cell lines than in human tumor cells. We have purified the enzyme from the murine colorectal cancer cell line Colon-26 and, based upon partial amino acid sequences, have cloned the full length gene (5). Then human UdRPase was identified from the human colorectal cancer cell line HCT116 by using the mouse UdRPase cDNA as a probe. The identity of the cloned gene was confirmed as being human UdRPase in several ways. First, the DNA sequence and the deduced amino acid sequence have high degrees of homology to those sequences for mouse UdRPase (Fig.2). Second, the recombinant protein, expressed in COS-7 cells, catalyzed the phosphorolysis of uridine specifically (Table 1). Finally, the activity of the enzyme was inhibited by a competitive inhibitor of UdRPase, 2,2'-anhydro-5-ethyluridine, (Table 1).

We have demonstrated that UdRPase mRNA is expressed in various kinds of tumor cell lines (Fig.3a), although the activity of UdRPase in various human tumor samples is very low as compared with that of TdRPase (data not shown). UdRPase expression is increased in HCT116 cells as well as in the murine colorectal tumor cell line Colon-26, in response to a mixture of the cytokines TNFα, IL-1α and IFNγ. The expression of the TdRPase gene is also up-regulated in human cancer cells by these cytokines (9).

In conclusion, we have cloned human UdRPase cDNA and shown that its expression is regulated at the mRNA level by cytokines and vitamin D3. Further

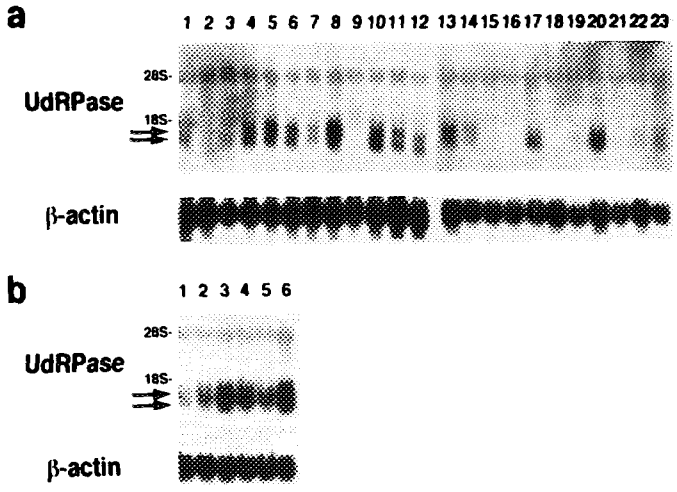

Fig.3. **Northern blot analysis of human uridine phosphorylase expression.** 20 mg of total RNA from 1: WI38, 2: WiDr, 3: HCT116, 4: DLD-1, 5: Lovo, 6: CXF280, 7: Colo201, 8: Colo205, 9: Colo320DM, 10: AGS, 11: KATO III, 12: MKN1, 13: MKN28, 14: MKN45, 15: ZR-75-1, 16: MCF-7, 17: MDA-MB-231, 18: HL60, 19: U937, 20: A549, 21: HepG2, 22: T-24 and 23: HeLa were used as samples.
b: 20 μg of total RNA from HCT116 cells treated with nothing (1), vitamin D3 (2), TNFα (3), IL-1α (4), IFNγ (5) and the mixture of TNFα, IL-1α and IFNγ (6) were used as samples.

studies of this gene should help us to determine the relative roles of UdRPase and TdRPase in human tumor cells.

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