Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 1-7

CLONING OF A FUNCTIONAL cDNA FOR HUMAN CYTIDINE DEAMINASE (CDD) AND ITS USE AS A MARKER OF MONOCYTE/MACROPHAGE DIFFERENTIATION

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Received November 23, 1992

Summary: We have identified a cDNA clone for human cytidine deaminase (EC 3.5.4.5) during an investigation which aimed at cloning novel gene expression products related to monocyte/macrophage differentiation. The derived amino acid sequence of the clone comprises 145 residues yielding a molecular mass for the polypeptide of 16.1 kDa and exhibits a nearly 50% homology to cytidine deaminase from Bacillus subtilis. Cytidine deaminase activity of the cloned sequence could be demonstrated in a prokaryotic expression system. The mRNA is highly expressed in granulocytes while expression is very low in fibroblasts, chondrocytes, monocytes, and T- as well as B-cell lines. The mRNA can be induced in monocytes, the monocytoid cell line U937 and the myeloblastic line HL 60 by the differentiation inducer calcitriol.

Cytidine deaminase (CDD) catalyzes irreversibly the hydrolytic deamination of cytidine, deoxycytidine and several of their therapeutically useful analogues to the corresponding uridine derivatives. Although the significance of the enzyme in pyrimidine metabolism is not completely elucidated, CDD is believed to function as a pyrimidine scavenger. (1). The enzyme has been characterized in E. coli (2) and several eukaryotic organisms (3 - 8). The CDD gene has so far only been identified in Bacillus subtilis and E. coli. (9 - 11). There is no homology between the two derived CDD peptide sequences of these two organisms. The human form of the enzyme was studied in partially purified preparations of different organ and cell sources including liver (12), spleen (13), granulocytes (14) and lymphocytes (15). The biochemical properties have been determined as follows:

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The abbreviations used are: CDD = Cytidine deaminase; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate.

0006-291X/93 $4.00

The active form most likely consists of 4 identical subunits with a total molecular mass of 52 kDa; the monomer has a molecular mass of about 14 kDa as estimated by SDS-PAGE (13). The subunits are not linked by disulfide bonds.

The immature monocyte-like cell line U937 acquires characteristics of differentiated monocytes upon long term stimulation with calcitriol [1-α,25-dihydroxyvitamine D3] (16). Our CDD clone was isolated by differential screening of a cDNA library prepared from mRNA of U937 cells after in vitro differentiation using the differentiation/activation inducers calcitriol and IFN-γ. Since the clone represented a stimulus induced mRNA, this study investigates the potential of CDD gene expression to serve as a differentiation marker for monocyte related cell lines as well as monocytes.

Materials and Methods

Materials: 1-α,25-dihydroxyvitamine D3 [calcitriol] was a gift from Hoffman-La Roche (Basel, Switzerland); IFN-γ was obtained from Bioferon (Laubheim, FRG). Radioactive nucleotides ([α-^{32}P]dATP and [α-^{32}P]dCTP) were obtained from Amersham (Braunschweig, FRG). Restriction enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories (Berlin, FRG). The λ-ZAP II cloning vector system was obtained from Stratagene (La Jolla, CA, U.S.A.). Biochemical reagents were bought from Sigma (Deisenhofen, FRG), Serva (Heidelberg, FRG) and Merck (Darmstadt, FRG). All cell lines were obtained from ATCC (Rockville, MD, U.S.A.).

Cell culture: Human monocytes and granulocytes were prepared from peripheral blood by Ficoll-Hypaque centrifugation followed by an adherence step on plastic surfaces or sedimentation in 6 % dextran T 500, respectively. For stimulation experiments the monocytes were incubated for 2 days either with 100 U/ml IFN-γ or 10 nM calcitriol in RPMI 1640 buffered with 20 mM Hepes and supplemented with 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % fetal calf serum.

Monocytoid and myeloblastic cell lines were cultured according to the recommended ATCC protocols and stimulated for 2, 3 or 4 days with 100 U/ml IFN-γ or 10 nM calcitriol or both.

Chondrocytes were obtained by digesting fetal chondyles with 0.1 % trypsin and subsequently with 0.1 % collagenase at 37° C. Fibroblasts were isolated by incubating connective tissue with 0.1 % trypsin for several hours at 37° C. After removal of debris the cells were allowed to recover overnight in HAMs F12 medium (Biochrom KG, Berlin, FRG) supplemented with 10 % fetal calf serum.

For preparation of total RNA the cells were in all cases washed with PBS and transferred immediately into Guanidium-SCN buffer.

cDNA library: A cDNA library was constructed from mRNA of U937 cells stimulated for 2 days and 4 days with a combination of 10 nM calcitriol and 100 U/ml IFN-γ. The cDNA was cloned into the EcoRI site of λ-ZAP II. After amplification 1 x 10^5 recombinant clones were differentially screened using radioactively labeled single stranded cDNA probes from unstimulated and stimulated U937 cells. Hybridizations, washings and exposure of the filters were carried out under standard conditions (17). The stringency wash was done in 0.5 x SSC/0.1 % SDS at 55° C for 45 min.

Northern hybridizations: Radiolabeled insert probes were prepared according to the random priming method (18). 10 µg of total RNA were run on a 1 % agarose gel containing 6 % formaldehyde. Hybridization and washings were carried out as described elsewhere (17). The stringency wash was done in 0.2 x SSC/0.1 % SDS at 65° C for 30 min.

DNA sequencing: Double stranded DNA sequencing was performed on both strands according to the method described by Sanger (19) using the Sequenase kit from United States Biochemical Corp. (Cleveland, OH, U.S.A.) following the suppliers protocol.

Results and Discussion

Identification of a cDNA clone for human CDD

U937 cells were differentiated into mature monocyte-like cells by 2 and 4 days of combined stimulation with 10 nM calcitriol and 100 U/ml IFN-γ. 1 x 10^5 recombinant clones from the cDNA library prepared from a pool of these cells were differentially screened using total cDNA probes from unstimulated and stimulated cells. 8 out of 150 clones which hybridized either predominantly or exclusively to the cDNA probe from stimulated cells were subjected to sequence analyses. Database searches with the derived amino acid sequences revealed that one of the clones, EA37 (Fig. 1), showed considerable homology to CDD from Bacillus subtilis. The insert exhibits an open reading frame ranging from base 1 to a TAG stop codon at position 437. The derived peptide consists of 145 residues with a total molecular mass of 16.1 kDa. The identity between the derived amino acid sequence of EA37 and CDD from Bacillus subtilis is about 49 %. In addition, 25 residues represent conservative substitutions yielding a similarity score of about 68 %. The identity of the coding regions on the nucleotide level is less significant (51 %). Significant homologies to CDD from E. coli as well as human adenosine deaminase, porphobilinogen deaminase and myodeaminase were not detected. Further computer analyses did not reveal potential glycosylation sites characterized by the sequences N-x-T and N-x-S and showed that the isoelectric point of the derived peptide was 5.95.

Primer extension experiments (not shown) as well as comparison of the molecular mass of the derived peptide and the experimentally determined molecular mass of the monomer (14 kDa) suggest that EA37 represents almost the complete peptide coding region. We therefore investigated functional expression of the cDNA using a procaryotic expression system. E. coli XL1-Blue cells were transformed with the pBluescript based expression plasmid p37-cdd1 which contains the entire available coding region including a part of the 3'-non translated region of EA37. CDD activity in sonication extracts of p37-cdd1 transformed XL1-Blue cells was about 30-fold higher than in cells bearing pBluescript or the original recombinant vector pEA37 which carries the insert in a wrong reading frame (data not shown). These data together with the observed

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      gcccagaagcgtcctgcctgcaccctgaagcctgagtgtgtc      42
hu    A  Q  K  R  P  A  C  T  L  K  P  E  C  V       14
bs                                    M  N  R
      cagcagctgctggtttgctcccaggaggccaagcagtcagcc      84
hu    Q  Q  L  L  V  C  S  Q  E  A  K  Q  S  A       28
bs    -  :  -  :  T  E  A  L  K  -  :  :  M  -
      tactgcccctacagtcactttcctgtgggggctgccctgctc     126
hu    Y  C  P  Y  S  H  F  P  V  G  A  A  L  L       42
bs    -  A  -  -  -  K  -  Q  -  -  -  -  -  -
      acccaggaggggagaatcttcaaagggtgcaacatagaaaat     168
hu    T  Q  E  G  R  I  F  K  G  C  N  I  E  N       56
bs    -  K  :  -  :  :  :  :  -  -  -  -  -  -
      gcctgctacccgctgggcatctgtgctgaacggaccgctatc     210
hu    A  C  Y  P  L  G  I  C  A  E  R  T  A  I       70
bs    -  A  -  S  :  C  N  -  -  -  -  -  -  :
      cagaaggccgtctcagaagggtacaaggatttcagggcaatt     252
hu    Q  K  A  V  S  E  G  Y  K  D  F  R  A  I       84
bs    F  -  -  -  -  -  -  D  T  :  -  Q  M  :
      gctatcgccagtgacatgcaagatgattttatctctccatgt     294
hu    A  I  A  S  D  M  Q  D  D  F  I  S  P  C       98
bs    -  :  -  A  -  T  P  :  P  .  :  -  -  -
      ggggcctgcaggcaagtcatgagagagtttggcacc.  aac     333
hu    G  A  C  R  Q  V  M  R  E  F  G  T  .  N      112
bs    -  -  -  -  -  -  -  :  S  -  :  C  -  K  :
      tggcccgtgtacatgaccaagccggatggtacgtatattgtc     375
hu    W  P  V  Y  M  T  K  P  D  G  T  Y  I  V      126
bs    V  I  -  V  :  -  N  L  :  -  Q  I  K  E
      atgacggtccaggagctgctgccctcctcctttgggcctgag     417
hu    M  T  V  Q  E  L  L  P  S  S  F  G  P  E      140
bs    -  -  -  :  -  -  -  -  -  :  A  -  :  S  -
      gacctgcagaagacccagtgacagccagagaatgcccactgc     459
hu    D  L  Q  K  T  Q  *                            145
bs    -  -  :  D  E  R  K  L
      ctgtaacagccacctggagaacttcataaagatgtctcacag     501
      ccctggggacacctgcccagtgggccccagccctacagggac     543
      tgggcaaagatgatgtttccagattacactccagcctgagtc     585
      agcacccctcctagcaacctgccttgggacttagaacaccgc     627
      cgccccctgccccacctttcctttccttcctgtgggccctct     669
      ttcaaagtccagcctagtctggactgcttccccatcagcctt     711
      cccaaggttctatcctgttccgagc    736
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Fig. 1. Nucleotide and deduced amino acid sequence of EA37. The human CDD sequence (hu) has been aligned to the CDD sequence of Bacillus subtilis (bs) using the UWGCG program "gap". Dashes indicate identical amino acids; double points refer to conservative substitutions; single points indicate gaps introduced by the program in order to achieve optimal alignment. The asterisk refers to the TAG stop codon.

homology to CDD of Bacillus subtilis clearly demonstrate that EA37 codes for a functional human cytidine deaminase.

Expression of CDD mRNA

Fig. 2 shows the expression of CDD mRNA in cells isolated from different sources. Expression is very high in granulocytes which is consistent with a high

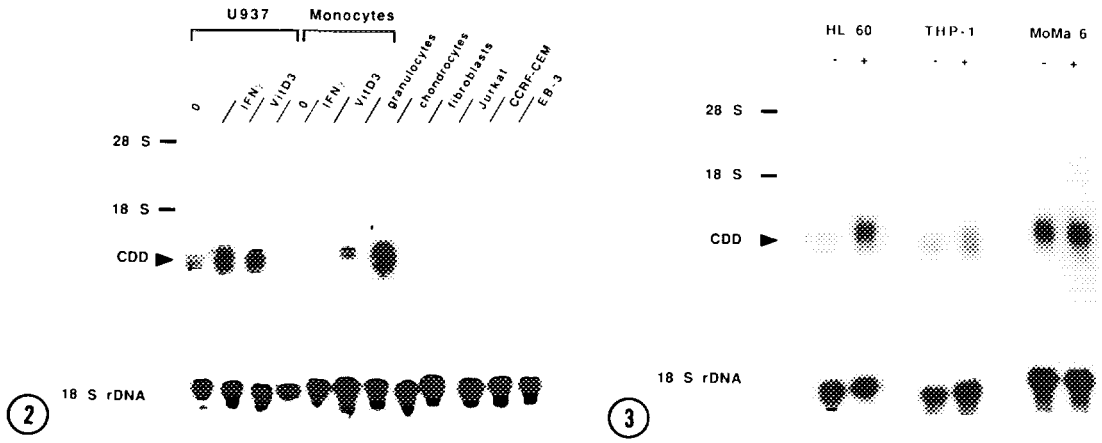

Fig. 2. CDD mRNA expression in different human cell types and inducibility in U937 monocytoid line and monocytes by 2 days of stimulation with 10 nM calcitriol and 100 U/ml IFN-γ. The blot was hybridized to an EcoRI/PstI fragment comprising the first 320 bases of EA37. It was reprobed with an 18 S rDNA clone in order to confirm comparable RNA loading. Positions of 28 S and 18 S rRNA are indicated.

Fig. 3. Inducibility of CDD mRNA expression in cell lines HL 60 (myeloblastic) THP-1 (monoblastoid) and MonoMac 6 (MoMa 6, monocytic). - = unstimulated, + = 3 days of stimulation with 10 nM calcitriol. The blot was probed and reprobed as described in the legend of Fig. 2. Positions of 28 S and 18 S rRNA are shown.

enzyme activity in these cells (14). CDD mRNA is almost undetectable in 10 µg of total RNA from chondrocytes and fibroblasts suggesting a minor role of the enzyme in these cells. The T-cell lines Jurkat and CCRF-CEM as well as an EBV transformed B-cell line also contained only very low detectable levels of CDD mRNA as did primary T-cells (not shown). CDD mRNA was also barely present in blood monocytes although it was inducible by 2 days of stimulation with 10 nM calcitriol which indicates that CDD mRNA expression may serve as a marker for monocyte/macrophage differentiation. To further evaluate this we checked CDD mRNA expression in various monocyte related cell lines and the myeloblastic cell line HL 60. CDD mRNA was almost undetectable in 10 µg of total RNA from HL 60 cells (Fig. 3). This line can be differentiated under appropriate conditions into granulocyte-like as well as monocyte-like cells (20) and is therefore considered to be arrested at a very early stage of the myelocytic/ monocytic differentiation pathway. The mRNA was strongly inducible in these cells by 3 days of stimulation with 10 nM calcitriol which is believed to initiate monocytic differentiation (20). This finding is consistent with the induction of enzyme activity by this stimulus (21). The monoblastic cell line THP-1 (Fig. 3) also showed a very low constitutive expression of CDD mRNA. The monocytoid line U937 (Fig. 2) expressed the mRNA at a considerably higher level than did THP-1 and HL 60 cells. In this line, but not in THP-1, CDD mRNA was inducible

by a factor of about 7 (densitometric estimation) by 2 days of stimulation with 10 nM calcitriol or 100 U/ml IFN-γ (Fig. 2). IFN-γ did not induce higher expression of CDD mRNA in HL 60 or THP-1 cells (not shown). The monocyte-like cell line MonoMac 6 represents a mature stage of monocyte differentiation (22) and showes a very high constitutive expression of CDD mRNA which could not be further increased by 3 days of stimulation with 10 nM calcitriol. In summary, constitutive CDD mRNA expression is very low in the myeloblastic cell line HL 60 and in the monoblastic line THP-1, intermediate in the immature monocytoid line U937 and high in the mature monocytic line MonoMac 6. Induction of the mRNA by calcitriol stimulated differentiation can be achieved in HL 60 and U937 but not in THP-1 and MonoMac 6. These data suggest that CDD mRNA expression can serve as a marker for monocyte/macrophage differentiation.

Cloning of the cDNA for human CDD offers the possibility to further investigate regulation of CDD gene expression and may also facilitate cloning of the gene from other mammalian species thereby allowing a detailed investigation of its significance for cellular nucleotide metabolism.

Acknowledgments

We thank Ms. Uta Meinke and Ms. Cornelia Rossmann for technical assistance and Dr. Scott Crowe and Dr. Jean Shearin (The Wellcome Foundation, Beckenham) for help with the manuscript.

References

1. Nygaard, P. (1986) Adv. Exp. Med. Biol. 195, 415 - 420.
2. Vita, A., Amici, A., Cacciamani, T., Lanciotti, M. and Magni, G. (1985) Biochemistry 24, 6020 - 6024.
3. Watanabe, K. A., Reichmann, U., Fox, J. J. and Chou, T-C. (1981) Chem. Biol. Interact. 37, 41 - 54.
4. Fanucchi, M. P., Watanabe, K. A., Fox, J. J. and Chou, T-C. (1986) Biochem. Pharmac. 35, 1199 - 1201.
5. Wisdom, G. B. and Orsi, B. A. (1969) Eur. J. Biochem. 7, 223 - 230.
6. Kidder, G. W. (1984) J. Protozool. 31, 298 - 300.
7. Ipata, P. L. and Cerciniani, G. (1978) Methods Enzymol. 51, 394 - 398.
8. Vita, A., Cacciamani, T., Natalini, P., Ruggieri, S., Santarelli, I. and Magni, G. (1988) Ital. J. Biochem. 36, 275 - 282.
9. Song, B. H. and Neuhard, J. (1989) J. Mol. Gen. Genet. 216, 462 - 468.
10. Valentin-Hansen, P., Holst, B., Josephsen, J., Hammer, K. and Albrechtsen, B. (1989) Mol. Microbiol. 3, 1385 - 1390.
11. Yang, C., Carlow, D., Wolfenden, R. and Short, S. A. (1992) Biochemistry 31, 4168 - 4174.
12. Chabot, G. G., Bouchard, J. and Momparler, R. L. (1983) Biochem. Pharmac. 32, 1327 - 1328.
13. Vita, A., Cacciamani, T., Natalini, P., Ruggieri, S. and Magn, G. (1989) Exp. Med. Biol. 253, 71 - 77.

14. Coleman, C. N., Stoller, R. G., Drake, J. C. and Chabner, B. A. (1975) Blood 46, 791 - 803.
15. Pérignon, J-L., Charléon, J., Leverger, G., Houllier, A-M., Thullier, L. and Cartier, P. H. (1985) 147, 67 - 73.
16. Testa, U., Ferbus, D., Gabbianelli, M., Pascucci, B., Boccoli, G., Louache, F. and Nguy Thang, M. (1988) Cancer Res. 48, 82 - 88.
17. Maniatis, T., Fritsch, E. F. and Sambrook, T. (1982) In Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, New York.
18. Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6 - 12.
19. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Nat. Acad. Sci. U. S. A. 74, 5463 - 5467.
20. Lyons, L. B. and Ashman, L. (1989) In Human Monocytes (Zembala, M. and Asherson, G. L. Eds.) pp. 59 - 70 Academic Press, Berkley.
21. Mejer, J. and Mortensen, B. T. (1988) Leukemia Res. 12, 405 - 409.
22. Ziegler-Heitbrock, H. W. L., Thiel, E., Futterer, A., Herzog, V., Wirtz, A. and Riethmüller, G. (1988) Int. J. Cancer 41, 456 - 461.