Cloning and sequencing of a cDNA encoding rat D-dopachrome tautomerase

Miao Zhang[a], Pierre Åman[a], Anders Grubb[b], Ioannis Panagopoulos[a], Annika Hindemith[d], Evald Rosengren[d], Hans Rorsman[c,*]

[a]*Department of Clinical Genetics, University Hospital, S-221 85 Lund, Sweden*
[b]*Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden*
[c]*Department of Dermatology, University Hospital, S-221 85 Lund, Sweden*
[d]*Department of Pharmacology, University of Lund, S-221 85 Lund, Sweden*

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Abstract An enzyme which converts D-dopachrome into 5,6-dihydroxyindole has recently been isolated from rat liver. Enzymatic D-dopachrome conversion has been observed in extracts from all tissues examined of several species, including man. We have now cloned and sequenced a 628 bp long cDNA encoding the enzyme provisionally called D-dopachrome tautomerase. The cDNA was isolated by 3′ and 5′ rapid amplification and cloning of cDNA ends (RACE) from rat liver cells using degenerate oligonucleotide primers, deduced from the N-terminal peptide sequence of D-dopachrome tautomerase. The cDNA contains an open reading frame encoding 118 amino acids. Edman degradation of intact and of trypsin degraded D-dopachrome tautomerase fragments gave information on and corroborated 67% of the deduced protein sequence. A homology search in the EST database found a human cDNA encoding a peptide sharing 66% homology with the rat enzyme. The rat D-dopachrome tautomerase shares 27% homology with the rat macrophage migration inhibitory factor (MIF).

Key words: D-Dopachrome tautomerase; Rat liver; PCR; cDNA cloning; Macrophage migration inhibitory factor

1. Introduction

The synthesis of melanin pigments giving color to the skin, hair and the eye is catalyzed by tyrosinase, which enzyme oxygenates tyrosine and oxidizes DOPA, the product formed at tyrosine oxygenation. Dopaquinone formed at the oxidation of DOPA is rapidly transformed into dopachrome [1]. Two enzymes present in melanin-forming cells (melanocytes) have been found to convert the colored compound L-dopachrome into the uncolored substance 5,6-dihydroxyindole-2-carboxylic acid by tautomerization [2,3]. Both enzymes are highly specific for L-dopachrome as substrate. We have recently found another enzyme, present not only in melanin-forming cells but in all tissues examined, that converts D-dopachrome into 5,6-dihydroxyindole [4]. The enzyme, first isolated from rat liver, was provisionally called D-dopachrome tautomerase because its D-dopachrome conversion activity, considered to involve a tautomerization with formation of a quinone methide [5], was used in monitoring its isolation [4]. We have now cloned and sequenced a cDNA encoding rat D-dopachrome tautomerase. Edman degradation and sequencing of intact and of trypsin degraded D-dopachrome tautomerase fragments gave information on and corroborated 67% of the deduced protein sequence.

2. Materials and methods

2.1. Protein isolation and determination of the N-terminal amino acid sequence

Isolation of D-dopachrome tautomerase from rat liver and analysis of its N-terminal amino acid sequence were performed as previously described [4].

2.2. Further protein structural analysis

The single polypeptide chain of D-dopachrome tautomerase was completely reduced and carboxymethylated prior to digestion with trypsin. 700 μg of D-dopachrome tautomerase and 14 μg of trypsin were incubated for 6 h at 37°C in 200 μl of 0.1 M ammonium bicarbonate, pH 8.0, and then lyophilized. The peptides of the digest were fractioned by reverse-phase HPLC using a 5 × 50 mm column of PepRPC HR 5/5 (Pharmacia), equilibrated in 0.1% (v/v) trifluoroacetic acid, run at room temperature with a flow rate of 1 ml/min and eluted with a 65 min gradient of acetonitrile from 0–100%. Peptide materials represented by peaks of 214 nm absorption were sequenced using an Applied Biosystems 470A sequencer with an on-line 120A PTH analyzer.

2.3. RACE

Poly-adenylated RNA was extracted from rat liver using poly(T) carrying magnetic beads as described [6]. First strand cDNA was synthesized with primer (pT)16-R1-R0 (5′AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT-3′) [7]. Three degenerate DNA primers, DDT I, DDT II, and DDT III, corresponding to peptide sequences of the N-terminal part of the protein, were designed (Fig. 1). The cDNA-product was used as template for the first round of PCR, which run with 0.5 μM of primer R0 (5′AAGGATCCGTCGACATC3′) [7] and 2.5 μM of the protein specific primer DDT I with a temperature profile of 1 min at 96°C, 1 min 42°C, and 3 min at 73°C. The second round PCR mixture contained 0.5 μM of R0 and 5 μM of DDT II as primers and the temperature profile was changed to 1 min 96°C, 2 min 42°C and 3 min 73°C. The third round of PCR run as round two with the primers R1 (5′-GACATCGATAATACGAC3′) [7] and DDT III (Fig. 1), and the annealing temperature at 45°C.

The 5′ end of the cDNA was isolated by a 5′ rapid amplification and cloning of cDNA ends (RACE) technique [8]. The first strand cDNA was ligated to an anchor adaptor (CTGGTTCGGC CCACCTCTGA AGGTTCCAGA ATCGATAGAT GTCCGGATGT) [9] which could be used as target sequences for two PCR primers. Two internal 3′ primers DDT4, TGCCAGGGCTCCAAGGGGAAG, and DDT5, GCTGTGGCTGCGGTTCTGCTC were designed based on sequencing data from the cDNA and a nested PCR was performed with these primers and the anchor adaptor primers to amplify the 5′ end of the specific cDNA.

*Corresponding author. Fax: (46) (46) 15-7887.

The sequence of rat D-dopachrome tautomerase has been deposited to the Protein Sequence Data Bank (EMBL), accession no. Z36980.

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N-terminal:

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Pro  Phe  Val  Glu  Leu  Glu      Thr      Asn LeuProAlaSerArgLeu-
CCC  TT(CT) GT(GC) GA(AG) CT(GC) GA(AG) AC(AGCT) AA(TC)
                DDT I

-ProAlaGlyLeuGluAsnArgLeuXxxAla  Ala      Thr      Ala      Thr      Ile-
                                 GC(CT) GC(CT) AC(ACT) GC(AGCT) AC(AGCT) AT(ATC)
                                          DDT III

-Leu Asp  Lys      Pro      Glu      AspArgValSerVal
  GA(CT) AA(AG) CC(AGCT) GA(AG) GA(TC)
     DDT III
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Fig. 1. N-Terminal peptide sequence and the degenerated oligonucleotide primers used for specific amplification of the D-dopachrome tautomerase cDNA.

2.4. Cloning of PCR products

PCR-products were purified with Magic PCR Preps DNA Purification System (Promega) and cloned into the *Srf*I site of the Bluescript vector using a pCR-Script SK(+) Cloning Kit (Stratagene). White colonies were tested for inserts using PCR with a M13 primer-set.

The Magic Minipreps DNA Purification System (Promega) was used for isolation of plasmids from bacteria cultures.

2.5. DNA sequencing

Sequencing was made with a T7 Sequencing Kit from Pharmacia according to the manufacturer's instructions. Homology searches and computer analysis of the sequences were performed with the tBLAST and tFASTA programs of the Wisconsin GCG package.

2.6. Northern blot

Northern blot was performed as described by Wennborg et al. [10] and a multi-tissue Northern blot filter was purchased from Clontech. Gel fractionated fragments from the cloned products were labelled as described by Feinberg and Vogelstein [11]. Probe hybridizations to Northern and Southern filters were performed according to Church and Gilbert [12].

3. Results

Edman degradation of intact, reduced and carboxymethylated, D-dopachrome tautomerase resulted in identification of 38 amino acid residues of an N-terminal sequence which was used to construct degenerate DNA primers (Fig. 1).

Edman degradations of peptides separated by reverse-phase HPLC of the trypsin digested, reduced and carboxymethylated single polypeptide chain of D-dopachrome tautomerase resulted in identification of clear sequence data for four peptides, one overlapping the N-terminal sequence with three amino acid residues (Figs. 1 and 2).

Agarose gel electrophoresis of the products from the first two rounds of PCR using DDT I and DDT II as 5′ primers showed a homogeneous smear extending from 1.2 kbp and down. The product from the third round of PCR showed five clear bands. The bands were cut out and cloned. Sequencing analysis showed that one clone, clone 35, from the 470 bp band contained sequences that corresponded to the amino acid sequence of the peptide fragments of D-dopachrome tautomerase (Fig. 2). Three more clones were isolated and sequence analysed with identical results.

The 5′ end of the cDNA was amplified and cloned by a 5′ RACE technique [7] with two reverse primers, DDT5 and DDT4, corresponding to the 3′ end of the cDNA. Five clones were sequence analysed and all showed an identical sequence overlapping the previously determined sequence with 131 basepairs. A full-length sequence could be assembled from 5′ and 3′ ends of the cDNA (Fig. 2). The putative peptide translated from the cDNA sequence contained sequences that were identical to the peptides previously determined by amino acid sequencing.

Clone 35 was labeled and hybridized to northern filters with total rat liver RNA and to a filter carrying RNA from different rat tissues. Two bands at approximately 600 and 2500 bases hybridized to the probe (Fig. 3).

When a homology search was performed in the databases for expressed sequence tags (EST), extensive homology was found with a recently isolated human cDNA clone (Gene Bank R62910) (Fig. 4). The human clone shows 66% identity and 82% homology of isofunctional residues with the rat D-dopachrome tautomerase. Rat D-dopachrome tautomerase also shares extensive homology with rat macrophage migration inhibitory factor, 27% identities and 53% homologies (Fig. 4).

4. Discussion

We have cloned and sequenced a 628 bp long cDNA encoding the rat D-dopachrome tautomerase. The cDNA contains an open reading frame encoding 118 amino acids. A homology search in the EST database found a human cDNA encoding a peptide that shares 66% homology with the rat enzyme (Fig. 4).

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                                                    tgaattcctggaacctcaactggct

cccactgctctctcggtgcagttaccgtttggcgatccctctcctgctaac

ATGCCGTTCGTTGAGTTGGAAACAAACTTGCCGGCTAGCCGCATACCCGCA
M  P  F  V  E  L  E  T  N  L  P  A  S  R  I  P  A
                                  10

GGGCTGGAGAACCGGTTGTGTGCGGCCACAGCCACCATCCTGGACAAACCC
G  L  E  N  R  L  C  A  A  T  A  T  I  L  D  K  P
         20                                  30

GAAGACCGCGTGAGCGTGACGATACGACCGGGCATGACCTTGTTGATGAAC
E  D  R  V  S  V  T  I  R  P  G  M  T  L  L  M  N
                  40                                  50

AAATCCACAGAGCCCTGCGCCCACCTCCTGATCTCTTCCATCGGTGTTGTG
K  S  T  E  P  C  A  H  L  L  I  S  S  I  G  V  V
                           60

GGCACCGCGGAGCAGAACCGCAGCCACAGCTCCAGCTTCTTCAAGTTCCTC
G  T  A  E  Q  N  R  S  H  S  S  S  F  F  K  F  L
      70                                  80

ACCGAGGAGCTGTCCCTGGACCAGGACAGGATCATTATCCGATTCTTCCCC
T  E  E  L  S  L  D  Q  D  R  I  I  I  R  F  F  P
               90                                  100

TTGGAGCCCTGGCAGATCGGAAAGAAAGGAACTGTTATGACGTTTCTG
L  E  P  W  Q  I  G  K  K  G  T  V  M  T  F  L
                        110

tgatggagacaaggaacgcagggcgtttgcttgagcctgtccagagccct

tccagagaggcctcctggcagatacgataccagatccctcttttgcataa

gtgtctgtgatctcactgacctgttttcctctcccccagcctcgtgaacg

agagagcaataaagaagagagcataatcccaaaaaaaaaaaaaaaaaa
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Fig. 2. cDNA and amino acid sequence of the rat D-dopachrome tautomerase. Translated cDNA sequences are given in capital letters. Amino acids that were determined by peptide sequencing are given in bold letters. The start methionine is not present in the mature protein. A polyadenylation signal sequence aataaa (underlined) is found 20 basepairs upstream of the poly(A) tail.

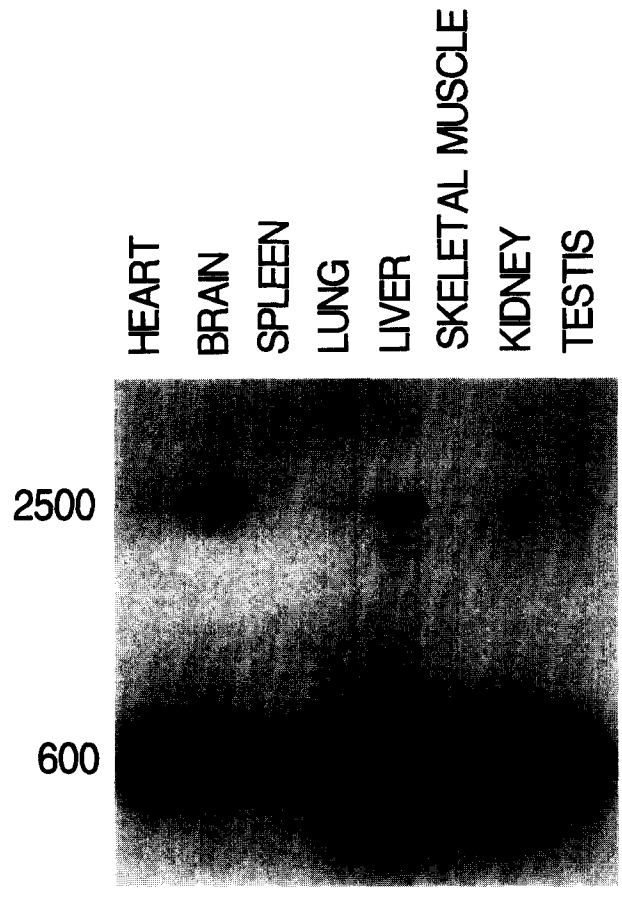

Fig. 3. Northern blot analysis of DDT expression. The analysed rat tissues are indicated in the figure. Sizes of the bands are given to the left in bases.

The human cDNA with homology to the cDNA of rat D-dopachrome tautomerase [13] may code for a protein giving D-dopachrome tautomerase activity in man [4].

The homology between rat D-dopachrome tautomerase and rat macrophage migration inhibitory factor is pronounced (Fig. 4B). Both proteins are of similar size, about 12 kDa. Two cysteine residues are present in D-dopachrome tautomerase, located at position 23 and 56 of the peptide, but MIF contains three cysteine residues [14,15], the first one at position 56 of the peptide and the second and third ones at positions 59 and 80.

The cysteine at position 56 is conserved in D-dopachrome tautomerase and in rat, mouse, human and chicken MIF, but the typical conserved MIF sequence, 56-CALC-59 differs in D-dopachrome tautomerase. Two potential *N*-glycosylation sites were found in D-dopachrome tautomerase at peptide positions 50 and 73 and rat MIF also has two potential glycosylation sites, one at peptide position 74 but the second one at position 109.

The presence of two transcripts at 2500 and 600 bases on northern blot implies that a different but related gene or two splicing variants exist (Fig. 3). The 5′ RACE, however, picked up only one band at 310 basepairs suggesting that no longer splicing variants exist of the rat D-dopachrome tautomerase transcript. The longer transcript seen in northern blots may therefore represent a transcript from a related but different gene.

The tautomerization of D-dopachrome was first observed in experiments on conversion of L-dopachrome of cultured melanoma cells when the D-isomer of dopachrome was used as the control substrate. Conversion of D-dopachrome can hardly be the true function of the enzyme as the presence of D-dopachrome in the organism is unlikely. The natural function of D-dopachrome tautomerase is still not known but it has been found in extracts of all organs examined, liver, kidney, spleen, brain, and heart, and in all animal species studied, man, rat, cow, chicken and fish ([4], and unpublished observations). The homology with the macrophage migration inhibitory factor (MIF) could suggest a role for D-dopachrome tautomerase in inflammation, since MIF is of importance in the inflammatory response. MIF has been studied for three decades in inflammatory reactions, but recent studies have shown that this lymphokine is not limited to being a product of stimulated T lymphocytes. MIF has thus been demonstrated in several tissues of many species [14–23]. The finding of a widespread distribution of MIF in different tissues has stimulated thoughts on additional autocrine roles of this protein and it has been suggested that MIF may be of importance in cell growth and differentiation [17,22].

Isolation of the D-dopachrome tautomerase made partial peptide sequencing possible and the sequence data enabled us to design degenerate primers for PCR amplification of the specific cDNA. The degenerate 5′ primers together with the highly non-specific poly(T) coupled 3′ primer gave enough specificity for a direct amplification of the D-dopachrome tautomerase cDNA by nested PCR-reactions. The results indicate that this procedure is a useful and fast method for isolation of cDNAs when only partial peptide sequences are known.

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DDT      1 MPFVELETNLPASRIPAGLENRLCAATATILDKPEDRVSVTIRPGMTLLM 50
R62910   1 MPFLELDTNLPANRVPAGXEKRLCAAAASILGKPADRVNVTVRPGLAMAL 50

DDT     51 NKSTEPCAHLLISSIGVVGTAEQNRSHSSSFFKFLTEELSLDQDRIIIRF100
R62910  51 SGSTEPCAQCSISSIGVVGTARDNRSHSAHFFEFLTKELAXGQ..ILFRF 98

DDT    101 FPLEPW 106
R62910  99 FPLESW 104
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B

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RATDDT   1 MPFVELETNLPASRIPAGLENRLCAATATILDKPEDRVSVTIRPGMTLLM 50
RATMIF   1 MPMFIVNTNVPRASVPEGFLSELTQQLAQATGKPAQYIAVHVVPDQLMTF 50

RATDDT  51 NKSTEPCAHLLISSIGVVGTAEQNRSHSSSFFKFLTEELSLDQDRIIIRF 100
RATMIF  51 SGTSDPCALCSLHSIGKIGGA.QNRNYSKLLCGLLSDRLHISPDRVYINY 99

RATDDT 101 FPLEPWQIGKKGTVMTFL 118
RATMIF 100 YDMNAANVGWNGSTFA   115
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Fig. 4. (A) Homology analysis (GCG program bestfit) between the rat DDT and human cDNA clone R62910 shows (62/93) 66% identities, (77/93) 82% homology of isofunctional residues. (B) Homology analysis between the rat DDT peptide and the rat macrophage migration inhibitory factor (RATMIF) [15], shows (32/115) 27% identities and (61/115) 53% homology of isofunctional residues.

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