Molecular characterization of a human tyrosinase-related-protein-2 cDNA Patterns of expression in melanocytic cells

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Pigmentation in mammals is under complex genetic control. Amongst the genes involved in this process, those encoding tyrosinase and the tyrosinase-related-proteins 1 and 2 have been well characterized and share a number of features. Recently, the murine tyrosinase-related-protein-2 gene was shown to encode dopachrome-tautomerase activity and was mapped to the slaty locus. Human tyrosinase and tyrosinase-related-protein-1 genes have been isolated and demonstrate a high degree of similarity with their murine counterparts. However, there has been limited data regarding the existence of a human homologue for tyrosinase-related-protein-2 and its relationship to the other tyrosinase-related proteins. In this study, we report the molecular isolation of a cDNA encoding a human homologue of the murine tyrosinase-related-protein-2/dopachrome tautomerase. We have characterized its expression in human melanocytic cells and have analyzed the relationship between dopachrome tautomerase and tyrosinase activities with the level of visible pigmentation in these cells. TYRP2 has been mapped to the chromosomal region 13q32, thus extending a region of synteny with mouse-chromosome 14.

Much recent progress has been made in the understanding of the molecular mechanisms that regulate melanogenesis. This process occurs in the melanosomes which are specialized subcellular organelles within the melanocytes. Melanin results from the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-Dopa), and the subsequent oxidation of L-Dopa to L-dopaquinone. Until recently, tyrosinase [1] was thought to be the only enzyme involved in this process. However, the many well-described mouse mutations which affect hair color had suggested that melanogenesis was the result of multiple interacting genes and regulatory factors [2]. The complexity of this process has been illustrated by the concomitant isolation of cDNA clones for tyrosinase and additional tyrosinase-related proteins. These molecules define a new family of proteins sharing structural primary sequence similarity. Moreover, some of the murine hair color phenotypes result from mutations in the tyrosinase and tyrosinase-related-protein genes, which have helped to distinguish these various pigmentation genes and the proteins that they encode.

One such protein, the enzyme dopachrome tautomerase, has been the subject of much controversy. This enzyme is involved in the conversion of dopachrome to eumelanin, and was named successively L-dopachrome conversion factor [3], L-dopachrome oxidoreductase [4] and L-dopachrome iso-

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Abbreviations. L-Dopa, 3,4-dihydroxyphenylalanine; PCR: polymerase chain reaction.

Enzymes. Tyrosinase (EC 1.14.8.1); dopachrome tautomerase (EC 5.3.2.3).
demonstrate dopachrome-tautomerase activity, strongly suggesting that this gene encodes dopachrome tautomerase.

MATERIALS AND METHODS

Cells

Human melanoma cell lines SK-MEL-19, SK-MEL-131 (clones 1–5 and 344), SK-MEL-188, and the murine melanoma cell line B16 have been previously described [11] and were maintained in minimum essential medium supplemented with 10% fetal calf serum. The human melanoma cell lines BEU, LND1, IGR3, SCL, HBL, and the murine melanoma cell line Cloudman S91, were cultivated in HAM F10 medium supplemented with 5% fetal calf serum. AZM, MNGHM, MNWC, MNKB were normal human melanocytes cultured in HAM F10 medium as described by Smit al et. [12]. The cell line BEU was the generous gift of Dr J. F. Doré (Institut National de la Santé et de la Recherche Médicale, Lyon) and IGR3 was kindly provided by Dr Aubert (Marseille). The cell line Cloudman S91 was a gift from Dr Abdel-Malek (Cincinnati, Ohio). SCC1 is a human lingual carcinoma cell line.

Isolation of human tyrosinase-related-protein-2 cDNA

Degenerate polymerase-chain-reaction (PCR) primers corresponding to homologous regions in the members of the tyrosinase-related-protein family (murine and human) were synthesized. These included the forward primer

\[ 5'\text{TACGAGGGA-GAGA-GAG} A3'\]

and the reverse primer

\[ 5'\text{TTCAGAGTCCTTTTGCAATNNNGGNC} 3'\].

These primers correspond to amino acids DDREXWP (forward primer, flanked by an XhoI site) and APIGHNR (reverse primer, flanked by a SstI site) in the different tyrosinase-related proteins. Total RNA was prepared from melanoma cells and first-strand cDNA was synthesized as described by reverse transcription [13]. 1 µl of the total 50-µl reverse-transcription reaction was subjected to amplification by PCR. Thirty cycles (94°C, 1 min; 45°C, 2 min; 74°C, 2 min) were performed in a Hybaid thermocycler. The amplification products were digested with PvuII (which has one cleavage site in the tyrosinase and tyrosinase-related-protein-1 cDNAs) and the products were electrophoresed on 0.8% agarose gels or on 5% non-denaturing polyacrylamide gels. DNA fragments were transferred to nylon membranes and hybridized with probes specific for human tyrosinase and tyrosinase-related-protein-1. Specific bands which did not hybridize were gel purified, labeled with \(\alpha-\text{P}dC\) using the random-priming method [14], and used to screen a cDNA library constructed from the melanoma cell line SK-MEL-19 [15]. Positive clones were analyzed and sequenced on both strands as previously described [15].

Northern-blot analysis

Total RNA was extracted and 10 µg was fractionated on 1% formaldehyde/agarose gels, and transferred onto GeneScreen membranes (Dupont). cDNA probes were labeled using the technique of random priming (USB) and the radiolabel \([\alpha-32P]dCTP\).

Determination of dopachrome-tautomerase activity

Prior to determination of dopachrome-tautomerase enzyme activity, cells (8.5×10⁶/ml) were lysed in Hanks’ balanced salt solution containing 1% Nonidet P40. 0.1 mM EDTA was added to prevent dihydroxyindole-carboxylic-acid formation due to metal ions [16]. After homogenization and sonication, cellular debris were eliminated by a 10-min centrifugation at 9000 g. Resulting lysates were immediately assayed by spectrophotometry using two different parameters. The absorbance decrease at 475 nm, due to L-dopachrome consumption, and the parallel absorbance increase at 308 nm, due to dihydroxyindole-carboxylic-acid formation were both followed. In both cases, dopachrome was prepared by the stoichiometric oxidation of L-Dopa by sodium periodate. 1 U of dopachrome tautomerase was defined as the amount of enzyme that produces 1 µmol of dihydroxyindole-carboxylic acid/min at 25°C [17]. Tyrosinase activity was determined using a modification [18] of the Pomerantz method [19], using L-[3,5-3H]tyrosine as substrate. 1 U of tyrosine-hydroxylase activity was defined as the amount of enzyme that catalyzes the hydroxylation of 1 pmol L-tyrosine/min at 37°C. Protein concentrations were determined using the Lowry assay [20]. The eumelanin content has been previously determined for the cell lines IGR3, LND1, HBL and SCL [21]; for the other cell lines, pigmentation was visually estimated by the intensity of the individual brown or black pellets.

Fluorescence in-situ hybridization

The tyrosinase-related-protein-2 cDNA insert of the clone 5B2 was used as a probe for in-situ hybridization. After labeling by nick translation with 5'-[N-[biotinyl-L-amino caproyl]-3-aminoallyl]-2'-deoxyuridine 5'-triphosphate (Bio-11-dUTP), the probe was purified over a Sephadex G-50 column. The biotinylated probe (100 ng/ml) was added to the hybridization mixture (50% formamide, 2× NaCl/Cit, 10% dextran sulfate, pH 7, 500 ng/ml sonicated salmon sperm DNA; 20× NaCl/Cit = 3 M NaCl, 0.3 M sodium citrate, pH 7), denatured and hybridized on the slide as described [22]. After an overnight incubation and post-hybridization washes, the biotinylated probe was detected by avidin–fluorescein isothiocyanate and the signal was amplified with additional treatments of biotinylated goat anti-avidin and avidin–fluorescein isothiocyanate. For the chromosomal localization, fluorescent R bands were obtained as described [23].

RESULTS

A third member of the human tyrosinase-related-protein family is detected by PCR

PCR primers were designed based on conserved protein sequences between the different tyrosinase-related proteins and tyrosinase. Using these primers, cDNAs were amplified from several human melanoma cell lines (SK-MEL 188, SK-MEL-19 and LND1), and in each case a single, well-defined 1-kb product was observed (data not shown). Human tyrosinase and tyrosinase-related-protein-1 each contain unique PvuII sites (at Ala299 in tyrosinase and at Ala292 in tyrosinase-related-protein-1), thus allowing for their discriminat-
The reactions generated two additional fragments which are not found in Fig. 1. Restriction digest of PCR products from members of the tyrosinase-related-protein family. PCR products from tyrosinase (Ty), tyrosinase-related-protein 1 (Trp-1) and the degenerate-primer (PCR) reactions were digested using PvuII. The degenerate-primer reactions generated two additional fragments which are not found in the tyrosinase-specific and TRP-1-specific reactions. Arrows indicate the specific TRP-2 amplified products.

Isolation and characterization of a cDNA encoding human tyrosinase-related-protein 2

A cDNA library from the human melanotic cell line SK-MEL-19 was screened using the 536-bp-specific fragment described above. Nine positive clones were isolated and each possessed the same restriction map, differing only in their overall lengths. The complete nucleotide sequence of the longest of these clones, termed 5B2, showed a single open reading frame encoding a 519-amino-acid polypeptide (Fig. 2). A leader sequence (residues -21 to -1) was assigned based on the rule of von Heijne [24]. Using the hydrophobicity pattern [25], a transmembrane domain could be predicted (residues 453–474). The 15 cysteine residues present in the mouse tyrosinase-related-protein-2 and in the other tyrosinase-related proteins [26] were conserved, and seven potential glycosylation sites could be predicted (NXS or NXT), in positions identical with the mouse tyrosinase-related-protein-2. Significantly, the cysteine residues at positions 85 and 88, which are potentially involved as iron-binding sites [27] are conserved in the murine tyrosinase-related-protein-2. Histidine residues, presumably involved in copper binding are also conserved. Sequence analysis and comparison with the mouse tyrosinase-related-protein-2 cDNA revealed similarity at the nucleotide (70%) and at the amino acid level (80%). Interestingly, a group of amino acids in the mouse sequence which contains the site of the slaty mutation (HYYSVRLDLLL, codon 173) was conserved in the human tyrosinase-related-protein-2. Based on these similarities, we designated the clone 5B2 as the human homologue for tyrosinase-related-protein-2. Amino acid sequence similarities with human tyrosinase-related-protein-1 and human tyrosinase were 46% and 40%, respectively (Fig. 3).

Expression of the human tyrosinase-related-protein-2 transcript

Tyrosinase-related-protein-2 expression was analysed using a panel of human melanocytic cells by Northern-blot analysis. In Fig. 4A, the specific transcript for tyrosinase-related-protein-2 is demonstrated at approximately 4.5 kb. This contrasts with the size of the murine transcript which is detected at approximately 3.0 kb (Fig. 4, lane 8). The difference in size between the transcript detected in Northern-blot analysis and the isolated cDNA for the human tyrosinase-related-protein-2 may indicate the presence of an extended untranslated region in the gene. Melanocytic cell lines can be placed into three groups based on the levels of tyrosinase-related-protein-2 transcripts. No transcripts were detected in the cell lines IGR3 (Fig. 4) and SK-MEL-131 (clone 1–5) (data not shown). A second group of cells showed low or moderate expression of TRP-2 transcripts [cell lines SCL, HBL and cell line BEU and normal melanocytes (AZM, MNGHM, MNWC, MNKB, data not shown)]. The third group [SK-MEL-19, SK-MEL-188, SK-MEL-131 (clone 344), Fig. 4 and LND1, data not shown] show relatively high levels of expression. No transcripts were detected in RNA from the human carcinoma cell line SCC1 (Fig. 4) or from human fibroblasts (data not shown). These data were also confirmed by PCR amplification of cDNA from these cell lines (data not shown). Fig. 4B shows the hybridization pattern with the actin control cDNA demonstrating equivalent RNA loading in each lane.

Dopachrome-tautomerase activity detected in human melanocytic cells in vitro

We have further analysed the relationship between the presence of tyrosinase-related-protein-2 transcripts and dopachrome-tautomerase activity. In these studies, the murine melanoma cell-line Cloudman S91 was used as a positive control. The dopachrome-tautomerase activity is presented as the increase of absorbance at 308 nm, which is a direct measure of the appearance of dihydroxy-indole-carboxylic acid. As shown in Table 1, activity can be detected in all the cells expressing the 4.5-kb transcript for tyrosinase-related-protein-2. Of particular significance is the high level of activity detected in SK-MEL-19 cells. However, there was not always a direct correlation between the level of dopachrome-tautomerase activity and the expression of the specific transcripts (for example, compare SK-MEL-188 and SK-MEL-19). Tyrosine-hydroxylase activity was additionally measured in this panel of cells and its activity did not correlate with that of dopachrome tautomerase. Moreover, there was no direct relationship between the presence of eumelanin and the dopachrome-tautomerase activity detected in these cells.

Chromosomal mapping by in-situ hybridization

Localization of the human tyrosinase-related-protein-2 gene (termed TYRP2 in accordance with the human nomenclature rules) was performed using non-radioactive hybridization with the 5B2 cDNA insert. Of the 40 metaphases examined, 26 (65%) demonstrated a specific signal, defined by two symmetrical yellow-green spots on both chromatids.
on the long arm of one chromosome, and 18 (45%) exhibited the double spots on two identical chromosomes (Fig. 5A). Double signals were not detected on any other chromosomal region. R-banding patterns allowed for the precise definition of the hybridization signals on chromosome 13 at 13q32 (Fig. 5B). This localization extends the region of conserved synteny between human chromosome 13 and mouse chromosome 14. A group of genes including Es-10, Htr2 and Rb map close together on mouse chromosome 14, about 25 cM proximal to Tyrp2 (Fig. 6). The corresponding human genes for Es-10, Htr2, and Rb localize to 13q14.1—14.2, proximal to the gene for Tyrp2. Cxn26/CXN26, which maps proximal to Htr2lRb in the mouse, has not been assigned to a specific band on human chromosome 13. However, based on the conservation presented in this study, we would predict that CXN26 will map proximal to 13q14.
**DISCUSSION**

In this study, we describe the isolation of a human cDNA clone encoding dopachrome tautomerase, mapping to chromosome region 13q32 and thus likely to be the homologue of the mouse tyrosinase-related-protein-2/dopachrome-tautomerase gene [9]. Previous studies had suggested the existence of a human protein cross-reacting with an antisera against murine dopachrome tautomerase [10] and the presence of a similar activity in human hair bulb [4]. This study indicates the existence of the enzyme dopachrome tautomerase in human cells.

The human tyrosinase-related-protein-2 cDNA isolated in this study encompasses the complete coding sequence of the protein. It demonstrates high similarity with its mouse counterpart at both the nucleotide and the amino acid level. It is interesting to note that despite this similarity, the mouse tyrosinase-related-protein-2 cDNA does not detect its human equivalent by Northern-blot analysis (unpublished results), although the human probe cross-reacts with the murine transcript. The sequence that we report in this study provides additional elements for the understanding of the evolution of this gene family. Each of the 15 cysteine residues present in the other tyrosinase-related proteins are conserved, as well as the histidine residues putatively involved in copper binding, and therefore in the biological activity of the enzyme.
Table 1. Summary of melanogenic properties of analyzed cell lines. Levels of TRP-2 transcripts were based on Northern-blot analysis (Fig. 4 and data not shown). –, no transcripts detected; +, moderate-level expression; ++, ++++, high-level expression. Pigmentation refers to the content of eumelanin, which was estimated visually for all cell lines. In addition, the eumelanin content for BEU, LND1, HBL and SCL have been determined (AHP/PTCA levels [21]). TH, tyrosine hydroxylase; DCT, dopachrome tautomerase; n.d., not determined.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TH activity</th>
<th>DCT activity</th>
<th>TRP-2 transcript level</th>
<th>Pigmentation level</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC1</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IGR3</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BEU</td>
<td>857.0 ± 402.0</td>
<td>3.8 ± 0.7</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LND1</td>
<td>621.6 ± 258.0</td>
<td>9.2 ± 3.4</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>HBL</td>
<td>694.4 ± 131.0</td>
<td>0</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>SCL</td>
<td>354.8 ± 2.7</td>
<td>3.9 ± 1.2</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>SK MEL 131 (clone 344)</td>
<td>n.d.</td>
<td>0</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>SK MEL 19</td>
<td>895.2 ± 337.0</td>
<td>29.1 ± 5.9</td>
<td>+++</td>
<td>+ + +</td>
</tr>
<tr>
<td>SK MEL 188</td>
<td>519.7 ± 63.0</td>
<td>1.9 ± 0.3</td>
<td>++</td>
<td>+ + +</td>
</tr>
<tr>
<td>S91</td>
<td>19.4 ± 9.4</td>
<td>20.8 ± 9.2</td>
<td>n.d.</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig. 5. Chromosomal localization of TYRP2 by non radioactive in-situ hybridization on human chromosomes. (A) Double spots are observed on the two chromosomes 13 at band 13q32. (B) R-banding pattern of the same metaphase.

Dopachrome tautomerase is also dependent on the presence of iron for optimum biological activity [27], and it is important to note the conservation of the cysteine residues potentially involved in iron binding. Other conserved regions of the molecule, such as the transmembrane domain, emphasize the probable importance of these regions in the function of tyrosinase-related proteins. In slaty mice, the amino acid Arg194 in the murine tyrosinase-related-protein-2 is substituted by a glutamine residue. The wild-type codon is only moderately conserved between the other tyrosinase-related proteins. However, it remains identical between the mouse and human sequences, suggesting that this site could play a role in the function of dopachrome tautomerase [9]. In addition, the C-terminus recognized by the serum anti-PEP-8 [10] is also well conserved, with only one amino acid difference (at position 488), replacing a glycine residue by a histidine. This similarity should allow the use of the anti-PEP-8 serum for biochemical characterization of the human tyrosinase-related-protein-2, including the analysis of its post-translational modifications, levels of glycosylation and intracellular processing.

In a previous study, we had shown that tyrosinase activity is not always correlated with the expression of the corresponding transcript and the amount of visible melanin produced in melanocytic cells [15]. In contrast, levels of tyrosinase-related-protein-1 mRNA and protein are directly correlated with the eumelanin content found in melanocytic cells [21, 28]. In light of these observations, it is interesting to note that although the presence of the tyrosinase-related-protein-2 transcript coincides with the detection of the dopachrome-tautomerase activity, there are individual variations in the levels of the enzyme activity when compared to those of the transcript in different cell lines. These data suggest the existence of possible post-transcriptional regulatory mechanisms, similar to those which have been reported for the tyrosinase transcript [28]. It should also be noted that no direct correlation exists between the expression of tyrosinase-related-protein-2 and the presence of visible melanin and/or activity of tyrosinase. These data differ from those recently reported by Kameyama et al. [29]. However, in their experiments, the authors used another approach including the use of clones derived from one cell line which could explain the discrepancies with our data.

Several studies have reported that melanocyte-stimulating hormone regulates dopachrome-tautomerase activity, although there are conflicting data concerning the precise effects that this hormone has on in-vivo transformation of dopachrome [4, 29, 30], in contrast to those reported for mouse tyrosinase activity [31]. Differential regulatory effects of melanocyte-stimulating hormone and other melanogenic
agents, such as theophylline, dibutyryl cAMP, and isobutyl methylxanthine would argue for specific mechanisms regulating the expression of the different tyrosinase-related proteins. This hypothesis is further substantiated by major differences in the gene structure and regulatory elements of tyrosinase and tyrosinase-related-protein-1 [28, 32, 33]. The structural organization of the mouse and human tyrosinase-related-protein-2 loci remain to be elucidated, as well as the regulatory elements which control their expression. Steel et al. [34] have shown that the tyrosinase-related-protein-2 gene is the first member of the tyrosinase-related-protein family to be expressed in the developing mouse embryo, suggesting a hierarchy of the transcriptional control of the tyrosinase-related-protein genes.

The availability of a cDNA clone for human tyrosinase-related-protein-2 will help to resolve questions regarding the biological activity of dopachrome tautomerase and its modulation by melanogenic agents. Indeed, transfection of our cDNA and characterization of the activity present in transfected cells, coupled with the use of specific antibodies, such as PEP-8, would provide direct evidence of the identity of the human protein encoded by the gene reported in this study. Transfection experiments performed with a full-length tyrosinase-related-protein-2 cDNA will also permit the analysis of potential interactions between tyrosinase, tyrosinase-related-protein-1 and tyrosinase-related-protein-2. It has been reported that these three melanogenic proteins exist as a high-molecular-mass complex in vivo inside the melanosomes [35]. It will be of particular interest to investigate the type of melanin produced in the presence (or absence) of the different genes. This kind of experimental model can be designed in an elegant manner with the use of transfecants, using the corresponding cDNAs.

Mouse mutations described at the pigmentation loci have provided invaluable information regarding their respective functions in melanogenesis. Complementary biochemical studies performed with specific antibodies have helped in the analysis of the tyrosinase-related-proteins biochemical structure and can be used as a direct measure of their in-vitro activity. It remains to be seen whether these melanogenic proteins require co-translation and specific interactions for efficient melanin synthesis. In this respect, transfection experiments are likely to represent an interesting in-vivo model, particularly for studying the functions and interactions of the melanogenic proteins, the pathway to melanosome biogenesis and the transport of the pigment vesicles to surrounding keratinocytes.

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