The 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase activity of human tyrosinase

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Melanin synthesis in mammals is catalysed by at least three enzymic proteins, tyrosinase (monophenol dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) and tyrosinase-related proteins (tyrps) 1 and 2, whose genes map to the *albino*, *brown* and *slaty* loci in mice, respectively. Tyrosinase catalyses the rate-limiting generation of L-dopaquinone from L-tyrosine and is also able to oxidize L-dopa to L-dopaquinone. Conversely, mouse tyrp1, but not tyrosinase, catalyses the oxidation of the indolic intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into the corresponding 5,6-indolequinone-2-carboxylic acid, thus promoting the incorporation of DHICA units into eumelanin. The catalytic activities of the human melanogenic enzymes are still debated. TYRP1 has been reported to lack DHICA oxidase activity, whereas tyrosinase appears to accelerate DHICA consumption, thus raising the question of DHICA metabolism in human melanocytes. Here we have used two different approaches, comparison of the catalytic activities of human melanocytic cell lines expressing the full set of melanogenic enzymes or deficient in TYRP1, and transient expression of *TYR* and *tyr* genes in COS7 cells, to demonstrate that human tyrosinase actually functions as a DHICA oxidase, as opposed to the mouse enzyme. Therefore, human tyrosinase displays a broader substrate specificity than its mouse counterpart, and might be at least partially responsible for the incorporation of DHICA units into human eumelanins.

Key words: melanin, melanocyte, melanogenesis, tyrosinase-related protein 1.

INTRODUCTION

Melanogenesis is the biochemical pathway responsible for melanin synthesis. In mammals, three related and highly similar metalloenzymes, tyrosinase (monophenol dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) and the tyrosinase-related proteins (tyrps) 1 and 2 are involved in the catalytic control of the process. Their cDNAs have been cloned and sequenced [1–6] and, in the mouse, the corresponding genes have been mapped to the *albino*, *brown* and *slaty* loci, respectively. The amino acid L-tyrosine is the metabolic precursor of the pigment. Tyrosinase catalyses the hydroxylation of L-tyrosine and the oxidation of the intermediate L-dopa to L-dopaquinone (L-DQ) [7]. L-DQ is a reactive intermediate that, in the absence of thiol compounds, spontaneously undergoes cyclization and further rearrangement, yielding L-dopachrome [8]. tyrp2 (dopachrome Δ^2,Δ^7-isomerase, EC 5.3.3.12), also called dopachrome tautomerase, catalyses the tautomerization of dopachrome into the more stable intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [9,10]. tyrp1 from mouse melanocytes has been reported to be a low-specific-activity tyrosinase isoenzyme with both tyrosine hydroxylase and dopa oxidase activities [11,12]. Moreover, this enzyme catalyses the oxidation of DHICA [13,14], thus promoting its incorporation into the eumelanin polymer. Therefore, in mouse melanocytes, the eumelanogenic pathway proceeds as shown in Scheme 1.

In spite of their extensive sequence similarities (40 % amino acid identities and approx. 70 % amino acid homologies), tyrosinase and tyrp1 display dramatic differences in their kinetic parameters and substrate specificities, at least in the murine system [11], where the enzymic capabilities of the melanogenic enzymes are better characterized. We have previously reported that purified tyrosinase and tyrp1 from mouse melanoma have different K_m values for both L-tyrosine and L-dopa substrates, as well as different requirements for the cofactor L-dopa needed in the hydroxylation of L-tyrosine. In addition, even though mouse tyrp1 is able to recognize DHICA and transform it into the corresponding quinone [13,14], mouse tyrosinase does not display significant DHICA oxidase activity.

On the other hand, the kinetic properties of the human melanogenic enzymes are less well characterized, probably due to the difficulty in obtaining enough biological material to attempt their large-scale purification. Some of the reported studies, performed either with relatively crude extracts or with purified enzyme preparations, have pointed out important similarities between the mouse and human melanogenic enzymes [15,16]. Therefore, it has been generally assumed that their kinetic behaviour should be very similar [17], and results obtained with the murine model are often extrapolated to human melanocytes. However, recent data suggest that the catalytic properties of the human and mouse melanogenic enzymes could display significant differences. For instance, affinity-purified human TYRP1 appears to possess tyrosine hydroxylase, but not dopa oxidase activity [18]. Interestingly, it has been reported recently that TYRP1 seems also unable to oxidize DHICA [19]. Accordingly, the human protein might share the tyrosine hydroxylase activity with the mouse enzyme, but might miss the *o*-diphenol and dihydroxyindole oxidase activities of murine tyrp1. On the other hand, DHICA monomers are actually incorporated into natural human melanin [20,21], and a DHICA converting activity has been attributed to human tyrosinase (TYROSINASE) expressed in fibroblasts [22]. However, this activity was determined from DHICA consumption experiments, and the reaction product was not characterized. These observations raise the possibility that

Abbreviations used: DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; L-DQ, L-dopaquinone; IQCA, 5,6-indolequinone-2-carboxylic acid; LFDM, low-fat dry milk; MBTH, 3-methyl-2-benzothiazolinone; tyrp, tyrosinase-related protein.

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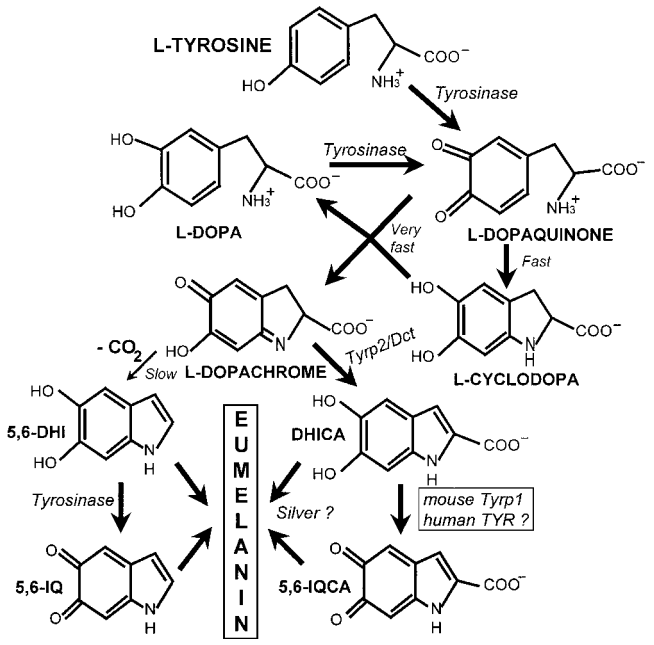

Scheme 1 Current view of the eumelanogenic pathway

The functions shown for the melanogenic enzymes, particularly tyrp1, are those demonstrated in murine melanocytes. Dct, dopachrome tautomerase; 5,6-DHI, 5,6-dihydroxyindole; 5,6-IQ, 5,6-indolequinone; 5,6-IQCA, 5,6-indolequinone-2-carboxylic acid.

TYROSINASE might recognize and oxidize DHICA, accounting for its incorporation into the melanin biopolymer. In any case, the catalytic properties of human and mouse tyrosinases could display subtle differences, but a comparison of the two enzymes, under identical experimental conditions, has not yet been performed.

Owing to the low availability of human melanogenic tissues, the purification of TYROSINASE and TYRP1 is hardly feasible. Alternative and complementary strategies to study their substrate specificities and kinetic behaviour are (i) comparison of the kinetics of the TYROSINASE and/or TYRP1 reactions in extracts from human melanoma cell lines expressing the complete set of melanogenic enzymes or defective in one or more of the proteins, a frequent situation in human melanoma cells [23–25] and (ii) expression of the corresponding genes in non-melanocytic cells, to avoid of possible interferences from other melanogenic proteins.

Using two melanoma cell lines expressing TYROSINASE, TYRP1 and dopachrome tautomerase, and one cell line defective in TYRP1, we have analysed the ability of the human enzymes to use DHICA as substrate. We show that extracts from human melanoma cells lacking TYRP1 are able to oxidize and metabolize DHICA. Moreover, at low concentration, DHICA acts as a cofactor for their tyrosine hydroxylase activities, whereas at higher concentrations, it inhibits tyrosine hydroxylation. Regarding the second approach, extracts of COS7 cells transiently transfected with the human *TYR* gene display DHICA oxidase activity, but those transfected with the mouse *tyr* gene do not. Overall, these results show that human TYROSINASE, as opposed to the mouse enzyme, is able to carry out DHICA oxidation and to promote the incorporation of carboxylated dihydroxyindoles into the eumelanin polymer.

EXPERIMENTAL

Reagents

The radioactive substrate L-[3,5-^{3}H]-tyrosine, specific activity 50 Ci/mmol, was obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). DHICA was a gift from Professor S. Ito (Fujita Health University, Toyoake, Japan). The specific αPEP1 and αPEP7 antisera, recognizing the C-terminal cytosolic extension of mouse tyrp1 and tyrosinase respectively, were a kind gift from Dr V. Hearing (National Institutes of Health, Bethesda, MD, U.S.A.). Electrophoresis and Western-blotting reagents and materials were from Bio-Rad (Hercules, CA, U.S.A.), unless otherwise specified. Reagents and plasticware for cell culture were obtained from either Nunc (Roskilde, Denmark) or Gibco (Gaithersburg, MD, U.S.A.). Other reagents were from Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany) or Prolabo (Barcelona, Spain).

Cell culture and preparation of crude solubilized extracts

Human melanoma cells were grown in Ham's F12 medium supplemented with 1 % streptomycin, 1 % penicillin, 1 % kanamycin, 2 % ultroser and 10 % fetal calf serum. The three lines, HBL, SCL and BEU, were originally established in the laboratory of Dr G. Ghanem (Laboratoire d'Oncologie et Chirurgie Experimentale, Brussels, Belgium) [23,24]. B16 mouse melanoma cells were cultured as described previously [26]. Cells were seeded in 75 cm^2 flasks at a density of 5×10^5 cells/flask, and allowed to grow to approx. 80 % confluence. Cells were harvested by trypsin treatment, washed twice with PBS, and solubilized in 10 mM sodium phosphate, pH 6.8, containing 1 % Igepal CA-630, 0.1 mM EDTA and 0.1 mM PMSF using approx. 1 ml of solubilization buffer/10^7 cells. The extracts were then centrifuged at 20000 ***g*** for 30 min to remove particulate material. The cleared supernatants were used for enzyme activity determinations. B16 mouse melanoma tyrosinase was purified from solid tumours maintained in C57/Bl mice as described previously [11,13].

Enzyme activity determinations

Tyrosine hydroxylase activity was determined by a radiometric method described elsewhere [27]. One unit was defined as the amount of enzyme catalysing the hydroxylation of 1 μmol of L-tyrosine/min, in the presence of a 50 μM concentration of the substrate and 10 μM L-dopa as cofactor.

Dopa oxidase activity was measured spectrophotometrically in the presence of 3-methyl-2-benzothiazolinone (MBTH), as described by Winder and Harris [28] with minor modifications, using final concentrations of L-dopa and MBTH of 3.0 and 3.25 mM, respectively.

DHICA oxidase activity was determined by either one of three complementary methods: (i) HPLC determination of DHICA consumption [13], (ii) spectrophotometric quantification of the formation of a coloured adduct of 5,6-indolequinone-2-carboxylic acid (IQCA) and MBTH [13,14], and (iii) spectral evolution of DHICA solutions in the UV/visible range in the presence of enzyme extracts [13]. DHICA was dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 0.1 mM EDTA to minimize spontaneous or metal-ion-catalysed oxidation.

Electrophoretic procedures

Analytical SDS/PAGE was performed as described in [11], in 12 % acrylamide gels, but without 2-mercaptoethanol and heat-

ing to preserve tyrosinase activity. Samples were mixed in a 2:1 ratio with sample buffer (0.18 M Tris/HCl, pH 6.8/15% glycerol/0.075% Bromophenol Blue/9% SDS), and electrophoresis was run at 4 °C. A highly sensitive and specific dopa oxidase activity stain was carried out by equilibrating the gels at pH 6.0 with 50 mM sodium phosphate buffer, followed by incubation at 37 °C in 1.5 mM L-dopa/4 mM MBTH, in 10 mM phosphate buffer, pH 6.8, from 15 to 30 min [29].

Immunochemical techniques

SDS/PAGE gels were run as described above, except for the presence of 2-mercaptoethanol in the sample buffer. Transfer to PVDF membranes (PolyScreen, NEN, Boston, MA, U.S.A.) was done in a semi-dry unit, and checked by Coomassie Brilliant Blue staining of the gel. For detection of tyrosinase with αPEP7, filters were blocked with 1% low-fat dry milk (LFDM) in PBS, maintained for 1 h at room temperature and incubated overnight with a 1:1000 dilution of αPEP7 in binding buffer 1 (10 mM Tris/HCl, pH 7.5/140 mM NaCl/5% LFDM). Membranes were washed three times for 5 min with washing buffer 1 (10 mM Tris/HCl, pH 7.5/140 mM NaCl/0.5% LFDM/0.5% Tween 20). Following incubation with a peroxidase-labelled secondary antibody (from Chemicon, Temecula, CA, U.S.A.; 1:2500 dilution in binding buffer 1), the filters were washed four times with washing buffer 1. For detection of TYRP1 using αPEP1 (final dilution of 1:100), a similar procedure was employed, but the antibody-binding buffer was PBS containing 1% BSA, 0.1 M NaCl and 0.2% Tween 20. Staining was done with a chemiluminescent substrate (Amersham Pharmacia Biotech), or with 3,3′-diaminobenzidine (0.6 mg/ml), $CoCl_2$ (0.03%) and H_2O_2 (0.001%).

Transient expression of human and mouse tyrosinase in COS7 cells

Expression constructs for human and mouse tyrosinases were prepared in the pcDNA3 expression vector (Invitrogen, Groningen, The Netherlands). For mouse tyrosinase, cDNA from B16 cells was obtained as described elsewhere [30], and amplified by PCR, using the proofreading *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) and the following primers, 5′-TGATGAATTCGAGAAA**ATG**TTCTTGGCTGT-3′ (forward) and 5′-GTTTTCTAGAATGT**TCA**CAGATGGCTCTGA-3′ (reverse), encompassing the start and stop codons (shown in bold), and containing added *Eco*RI and *Xba*I restriction sites (underlined). Amplification reactions yielded two products of different sizes that were cloned into pBluescript KS II (Stratagene, Heidelberg, Germany) using the restriction sites mentioned above. The identity of the products was verified by complete sequencing. The major band, of higher molecular size, was identical with the published wild-type sequence of the mouse enzyme ([31], GenBank accession number D00440). The minor band, of smaller size, corresponded to an alternative splicing variant whose occurrence has been reported by others [32]. This form, called Δ3*tyr*, lacks exon 3, between positions 1037 and 1184 of the coding sequence, thus generating a frameshift and a premature stop codon. Since the corresponding 390-amino-acid product is enzymically inactive due to the lack of the second copper-binding site, it was used as a negative control for all determinations of enzyme activity in transfected cells. For human TYROSINASE, the pMEL34 construct [33], obtained from the ATTC (Rockville, MD, U.S.A.) was used as target in PCR reactions using primers 5′-GGACAAGCTTAAGAATGCTCCTGGCTGTTT-3′ (forward) and 5′-CAGGTCTAGAGGCCCTACTCTATTGCCTA-A-3′ (reverse), encompassing the complete reading frame of the *TYR* gene and containing added *Hin*dIII and *Xba*I restriction sites (underlined) for cloning into pBluescript. The cloned product was sequenced and found to match exactly the sequence published for the *TYR* gene (GenBank accession number M27160) except for a C/A change at position 575 that corresponds to an already identified polymorphism [34]. The *tyr* (mouse, full-length tyrosinase), Δ3*tyr* (mouse, inactive alternatively spliced tyrosinase) and *TYR* (human, full-length tyrosinase) genes were subcloned into pcDNA3, and used to transfect COS7 cells with the Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were trypsin-harvested 24 h after transfection and processed for enzyme activity determinations or Western blotting as described above.

RESULTS

Melanogenic activities in TYRP1-positive and -negative human melanoma cells

The tyrosine hydroxylase, dopa oxidase and dopachrome tautomerase activities in three human melanoma cell lines (BEU, HBL and SCL) are shown in Figure 1(A). All the cell lines displayed significant levels of the three activities. Since BEU, HBL and SCL cells have been reported to express the *TYR* gene, but Northern-blot analysis of BEU cells suggests them to be TYRP1-negative [23], we wished for confirmation of these data at the protein and mRNA levels. The occurrence of enzymically active TYROSINASE protein was analysed by non-reducing SDS/PAGE followed by dopa oxidase activity stain [29]. As shown in Figure 1(B), human melanoma cells yielded a single tyrosinase activity band, whose electrophoretic mobility was identical in all cases, but markedly lower than the one of purified B16 melanoma tyrosinase. Expression of TYRP1 protein was analysed by Western blotting, with the mouse tyrp1-directed αPEP1 antiserum (Figure 1C). This antiserum reacts with an epitope located in the C-terminal pentadecapeptide of tyrp1. Although αPEP1 has been shown by others to be specific for the mouse enzyme, the conservation of 10 out of 15 amino acids between the mouse and the human C-terminal sequences appeared to confer on the antiserum a significant affinity for the human protein. Therefore, the antiserum could be used to detect TYRP1, under high-concentration conditions, to compensate for the lower affinity as compared with the mouse protein. αPEP1 reacted with a protein of approx. 75 kDa in extracts from SCL and, to a lesser extent, HBL cells, but not from BEU cells or human fibroblasts, thus proving the specificity of antibody binding under the experimental conditions employed to detect the human protein. Lack of expression of TYRP1 in BEU cells was further confirmed by reverse transcriptase PCR, using primers designed to amplify the complete coding sequence of the gene (Figure 1D). No amplification products were obtained with cDNA synthesized from total RNA extracted from BEU cells. However, an amplicon of the expected size (about 1.6 kb) was detected using cDNA from HBL cells as a positive control. Therefore, it is clear that BEU cells possess tyrosine hydroxylase, dopa oxidase and dopachrome tautomerase activities in the absence of TYRP1.

DHICA consumption by human melanoma cell extracts proceeds with oxidation and accelerated melanin formation

We compared the TYRP1-positive HBL and SCL cells, and the TYRP1-negative BEU cells, for their ability to metabolize DHICA. Extracts from each cell line were incubated at 37 °C with 0.5 mM DHICA, under conditions of comparable catalytic

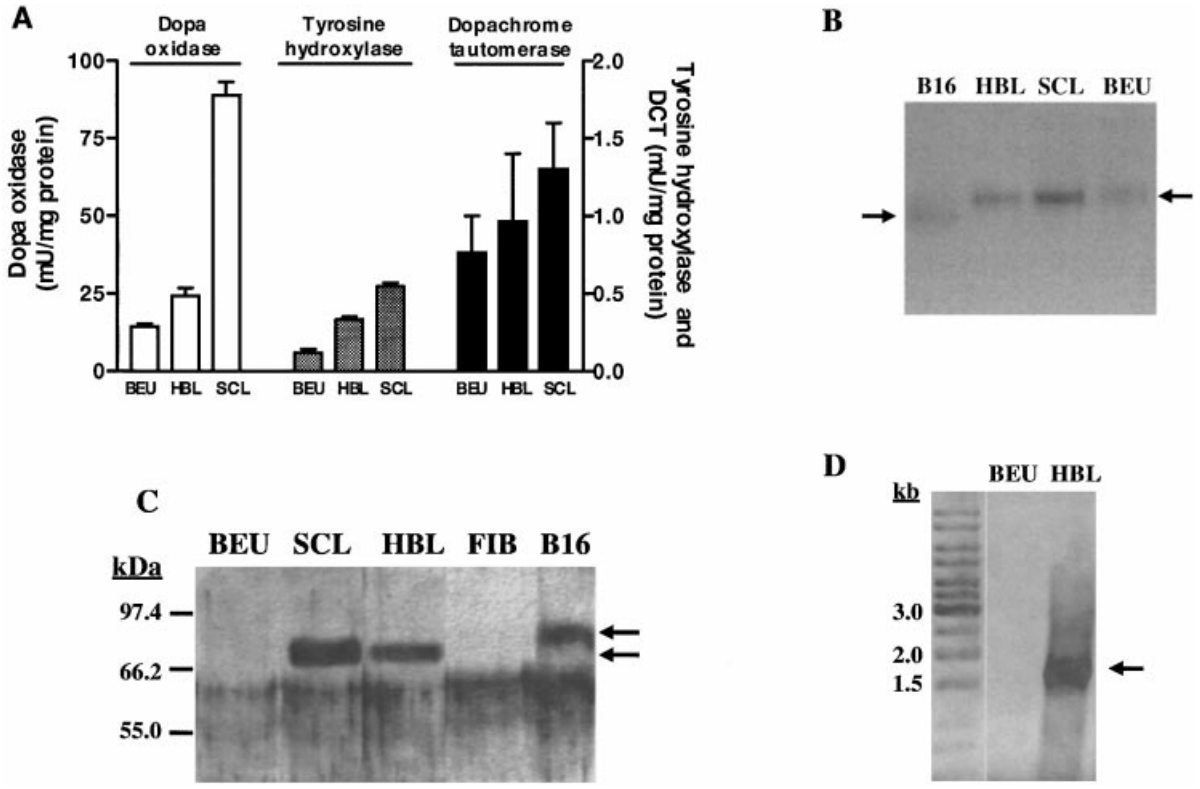

Figure 1 Melanogenic activities and enzyme expression in three human melanoma cell lines

(**A**) Melanogenic activities. DCT, dopachrome tautomerase. Results are the means ± SD from three independent assays with different cell extracts, each performed in duplicate. (**B**) Electrophoretic profile of purified tyrosinase (from B16 mouse melanoma) and the human enzyme from HBL, SCL and BEU melanoma cells. Crude extracts (30 μg of protein/lane) were resolved by non-reducing SDS/PAGE and stained for tyrosinase activity [29]. The left-hand arrow indicates the activity band corresponding to tyrosinase, whereas the right-hand arrow points to the activity band observed in the human melanoma cell extracts. (**C**) Western-blot analysis of TYRP1 expression in human melanoma cells. TYRP1 was detected with αPEP1 as primary antibody. The protein load was approx. 30 μg/lane. Molecular-mass standards are shown on the left. Arrows (lower and upper respectively) pointing to the human- and mouse-specific bands highlight the different electrophoretic mobilities of each protein. B16 and FIB designate crude extracts from mouse B16 melanoma cells and human fibroblasts, employed as positive and negative controls, respectively. (**D**) Lack of expression of the *TYRP1* gene in BEU cells. cDNA from BEU and HBL cells was amplified using primers encompassing the complete coding sequence of the *TYRP1* gene, and designed to yield an amplification product of 1644 bp. An aliquot of the amplification reaction was electrophoresed in a 1% agarose gel and stained with ethidium bromide. The arrow points to the single specific amplification product obtained.

dopa oxidase activity, and residual levels of DHICA were determined at different reaction times by HPLC. DHICA consumption by BEU extracts was low but detectable and significantly different ($P < 0.001$) from the blank (Figure 2). The HBL and SCL cell lines, expressing the complete set of melanogenic enzymes, exhibited a higher DHICA consumption activity. These data prove that DHICA is metabolized by human melanoma cell extracts, either in the presence or absence of TYRP1, but do not inform as to the nature of the reaction products. Therefore, experiments were carried out to establish whether DHICA consumption proceeds by oxidation and incorporation into melanins.

The evolution of DHICA solutions in the presence of human melanoma cell extracts was followed spectrophotometrically. Extracts from the three human cell lines accelerated eumelanin formation from DHICA, as measured by an increase in absorbance at 550 nm (Figure 3A), a wavelength indicative of dihydroxyindole dimers and pigment formation [35]. For comparable amounts of extract, the effect was faster for SCL cells, and comparable for BEU and HBL cells. In all cases, a lag phase was detected. This might correspond to initial trapping of the DHICA-derived quinone, IQCA, by thiol groups and other reactive nucleophiles present in the extract that would prevent dimer formation until they are exhausted. The decay in absorbance at 550 nm observed for SCL extracts after long incubation times is probably due to an accelerated progression of dihydroxyindole dimers to oligomers and polymeric species, resulting from the high melanogenic activity found in this cell line (see Figure 1A). This progression causes a darkening of the solution, and renders the absorbance peak centred at 550 nm flatter and lower [35]. Comparable catalytic amounts of purified B16 melanoma tyrosinase did not accelerate the spectral evolution of DHICA solutions (results not shown). This inability of mouse tyrosinase to accelerate DHICA evolution is fully consistent with previous reports from our group [13].

Although melanin formation should proceed through the oxidation of the corresponding precursors, we wished for direct confirmation of IQCA production by using the quinone-trapping reagent MBTH [13,14,28]. This experiment was performed with BEU cells to avoid possible interference from TYRP1. Figure 3(B) shows that BEU extracts catalysed the formation of a coloured adduct, in the presence of DHICA and MBTH. The absorption spectrum of this product was very similar to the one of the DQ-MBTH adduct [13,28]. Overall, these results prove that DHICA can be oxidized and incorporated into a melanin polymer by human melanocyte extracts completely lacking

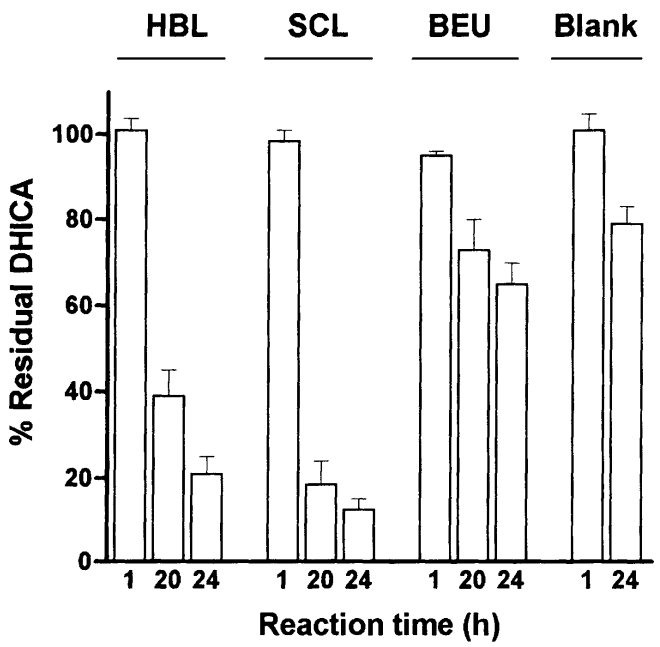

Figure 2 HPLC demonstration of DHICA consumption by human melanoma cell lines

Reaction mixtures containing 0.5 mM DHICA and amounts of extracts from HBL, BEU and SCL cells with comparable dopa oxidase activities (1.8, 1.1 and 1.0 m-units respectively) were incubated at 37 °C. The residual concentration of DHICA was analysed by HPLC at the times shown. A blank performed in the absence of cell extract is also shown to illustrate relative DHICA stability.

TYRP1, yet they do not demonstrate that TYROSINASE is the enzyme responsible for DHICA oxidation.

DHICA is recognized by TYROSINASE from human melanoma cell extracts

In order to obtain further evidence proving that DHICA is actually recognized by TYROSINASE, we analysed its ability to act as a cofactor and/or an inhibitor of tyrosine hydroxylation, using extracts from HBL and BEU cells as a source of enzyme. First, tyrosine hydroxylase activity was measured in the presence of 10 μM L-dopa as cofactor, and of comparatively high concentrations of DHICA. As shown in Figure 4, the tyrosine hydroxylase activity of extracts from both cell lines was decreased in the presence of DHICA concentrations higher than 250 μM. The inhibition was concentration-dependent and even higher for the TYRP1-negative BEU cells.

On the other hand, it is well known that the tyrosine hydroxylation reaction catalysed by tyrosinases from different sources displays a prolonged lag phase in the absence of appropriate cofactors [7,11,12]. The ability of L-dopa (either exogenous or generated *in situ* by disproportion between L-DQ and L-cyclodopa, as shown in Scheme 1) to act as a cofactor is attributed to the recruitment of *met*-tyrosinase to the active *oxy* form, upon oxidation of the diphenol [36]. Therefore, dihydroxy compounds serving as substrates for tyrosinase should be able to substitute L-dopa as a cofactor for the tyrosine hydroxylase reaction. According to this rationale, we measured the rate of tyrosine hydroxylation by human melanoma cell extracts in the presence of DHICA as putative cofactor, using reaction mixtures devoid of L-dopa and with various concentrations of DHICA. The results were compared with those found in standard conditions, in the presence of 10 μM L-dopa and in the absence of DHICA (Table 1). As expected, extracts from the three cell lines exhibited very low tyrosine hydroxylase activity in the absence of DHICA or L-dopa. In the presence of DHICA, the tyrosine hydroxylase activity increased in a concentration-dependent manner. The ability of DHICA to serve as a cofactor was similar for the three cell lines, irrespective of the presence or absence of TYRP1. However, DHICA was less efficient than L-dopa, since a 100 μM concentration of the dihydroxyindole only achieved about 40% of the activity obtained with 10 μM dopa. Higher

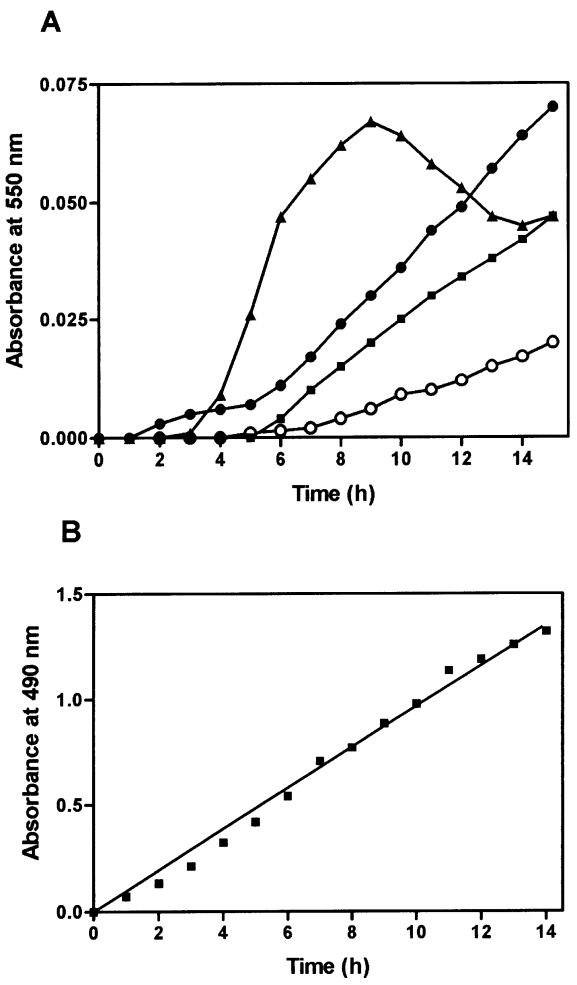

Figure 3 Spectrophotometric analysis of DHICA evolution in the presence of human melanoma cell extracts

(**A**) Melanin production from DHICA. The absorbance at 550 nm of DHICA solutions (150 μM final concentration) in 10 mM phosphate buffer, pH 6.0/0.1 mM EDTA, in the presence of human melanoma cells extracts, was measured at different times in a double-beam spectrophotometer at 37 °C, against a blank containing an identical amount of extract but no DHICA. ▲, SCL; ●, BEU and ■, HBL cell extracts; ○, blank obtained by adding to the DHICA solution a volume of solubilization buffer identical with the volume of enzyme extracts. The enzymic activities present in the reaction media, in terms of dopa oxidase activity, were 1.1, 1.2 and 2.0 m-units for SCL, BEU and HBL cells respectively. (**B**) BEU extracts catalyse the oxidation of DHICA to IQCA. The visible spectra of DHICA solutions (800 μM final concentration) in 10 mM phosphate buffer, pH 6.0/0.1 mM EDTA/2 mM MBTH, were serially recorded at 1 h intervals, in the presence of 1.15 m-units of dopa oxidase activity. The increase in absorbance at 490 nm, corresponding to the absorption peak of the MBTH-IQCA adduct, is shown. The reference cuvette contained an identical solution, except that the enzyme extract was replaced by an equivalent amount of protein from COS7 cells transfected with the inactive Δ3*tyr*.

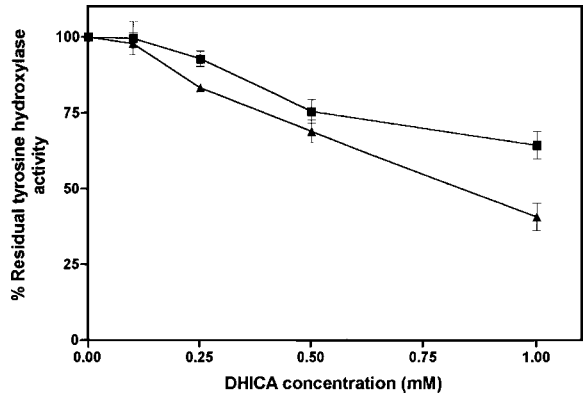

Figure 4 Inhibition by DHICA of the tyrosine hydroxylase activities of extracts from TYRP1-positive (HBL, ■) or -negative (BEU, ▲) human melanoma cells

The tyrosine hydroxylase activities of extracts from HBL (■) or BEU (▲) were measured in the presence of 10 μM L-dopa as cofactor, 50 μM L-tyrosine and increasing concentrations of DHICA. Results (means ± range, $n = 2$) are expressed as residual activity with respect to controls performed in the absence of DHICA.

Table 1 Ability of DHICA to act as cofactor of the tyrosine hydroxylase activity of human melanoma cell extracts

The tyrosine hydroxylase activity of human melanoma cell extracts was measured in the presence of 50 μM L-tyrosine and of the indicated concentrations of DHICA, but in the absence of L-dopa. Results are expressed as a percentage of the activity achieved with 10 μM L-dopa as cofactor, which was determined in parallel assays, in the absence of DHICA (means ± range, $n = 2$). Incubations were performed at 37 °C for 90 min.

DHICA (μM)	Maximal tyrosine hydroxylase activity (%)		
	BEU	HBL	SCL
0	5.4 ± 3.3	9.8 ± 3.2	3.1 ± 2.3
10	10.2 ± 5.3	19.5 ± 4.3	Not determined
100	32.5 ± 8.7	40.3 ± 7.5	39.5 ± 9.2

concentrations of DHICA were not tested, since, as shown above, they inhibit the rate of tyrosine hydroxylation.

DHICA is oxidized by TYROSINASE transiently expressed in COS7 cells

The results shown thus far prove that the TYRP1-negative BEU cells are able to catalyse DHICA consumption, most likely by an oxidative mechanism, and that DHICA is able to interact with TYROSINASE. These observations strongly suggest that TYROSINASE is the enzyme responsible for DHICA consumption. However, further possibilities, like the involvement of other melanogenic protein(s) cannot be ruled out. In fact, evidence has been presented by others pointing to a possible role of the *silver* locus protein in DHICA metabolism [37,38]. In an attempt to unambiguously demonstrate the ability of TYROSINASE to transform DHICA, and to characterize the reaction product, we transfected COS7 cells with the human *TYR* and mouse *tyr* genes.

Efficient expression of the TYROSINASE and tyrosinase proteins was ascertained by determination of the tyrosine hydroxylase and dopa oxidase activities of cell extracts and by Western blotting, using the tyrosinase-directed αPEP7 as primary antiserum (Table 2 and Figure 5A). Extracts from *tyr*-transfected cells displayed two major immunoreactive bands with apparent molecular masses of 52 and 63 kDa, and two minor bands of higher sizes, approx. 147 and 169 kDa. The 52 and 63 kDa bands probably correspond to the *de novo* form and mature, glycosylated form, respectively. The higher-molecular-mass forms are, most probably, aggregation products. The presence of aggregated forms of mammalian tyrosinases in SDS/PAGE has already been reported [11,17].

Table 2 Melanogenic activities in COS7 cells transiently transfected with the human (*TYR*) and mouse (*tyr*) genes

Results are the means ± range for two independent transfection experiments. All measurements were performed under the standard conditions described in the text with blanks performed with an amount of total protein equivalent to that of the samples, but from COS7 cells transfected with the enzymically inactive $\Delta 3$*tyr*. DHICA oxidase units were calculated from the ΔA_{490} in the presence of MBTH, by assuming a molar absorption coefficient for the IQCA-MBTH adduct identical with the one of DQ-MBTH [13].

Gene	Tyrosine hydroxylase (μ-units/mg of protein)	Dopa oxidase (m-units/mg of protein)	DHICA oxidase (μ-units/mg of protein)
TYR	98 ± 4	5.6 ± 0.2	88 ± 8
tyr	1110 ± 50	60 ± 3	Not detectable

Conversely, extracts from *TYR*-transfected cells showed a single 58 kDa band. Its lower intensity, as compared with the mouse gene product, was reproducibly observed in two independent transfection experiments. This might reflect a lower transfection efficiency, a less efficient translation, and/or a lower affinity of the tyrosinase-directed αPEP7 towards the human enzyme. In any case, COS7 cells transfected with the *TYR* gene displayed sufficient enzymic activity levels for accurate analysis (Table 2) within the range of those reported for B16 melanocytes [11,26].

Spectrophotometric analysis of DHICA evolution demonstrated that crude extracts from cells transfected with the human gene accelerated DHICA disappearance. The product formed was IQCA, as shown by formation of a coloured adduct with MBTH, monitored at 490 nm (Figure 5B). Again, mouse tyrosinase failed to oxidize DHICA to IQCA, according to the MBTH assay, even if present in catalytic amounts more than 10-fold higher than TYROSINASE, in terms of dopa oxidase activity, thus confirming previous reports [13,14]. Finally, the tyrosine hydroxylase activity of TYROSINASE, measured with a 10 μM concentration of the cofactor L-dopa, was inhibited by DHICA in a concentration-dependent manner (Figure 5C). This inhibition was even more potent than for human melanoma cell extracts. Surprisingly, a similar inhibition was also observed for the mouse protein.

DISCUSSION

Natural eumelanins are heterogeneous polymers containing monomeric units derived from both DHICA and 5,6-dihydroxyindole (DHI), the product of the spontaneous decarboxylation of dopachrome [39]. The relative proportions of DHICA-derived carboxylated units and DHI-derived decarboxylated monomers vary widely from one type of natural pigment to another [20,40]. Although rodent pigments seem richer in DHICA, the contents of these units in human pigments is nonetheless high, accounting for up to 20% of the total monomers [20]. The presence of a carboxy group in DHICA-derived monomers is believed to have a strong effect on the properties of the pigment. DHICA-rich

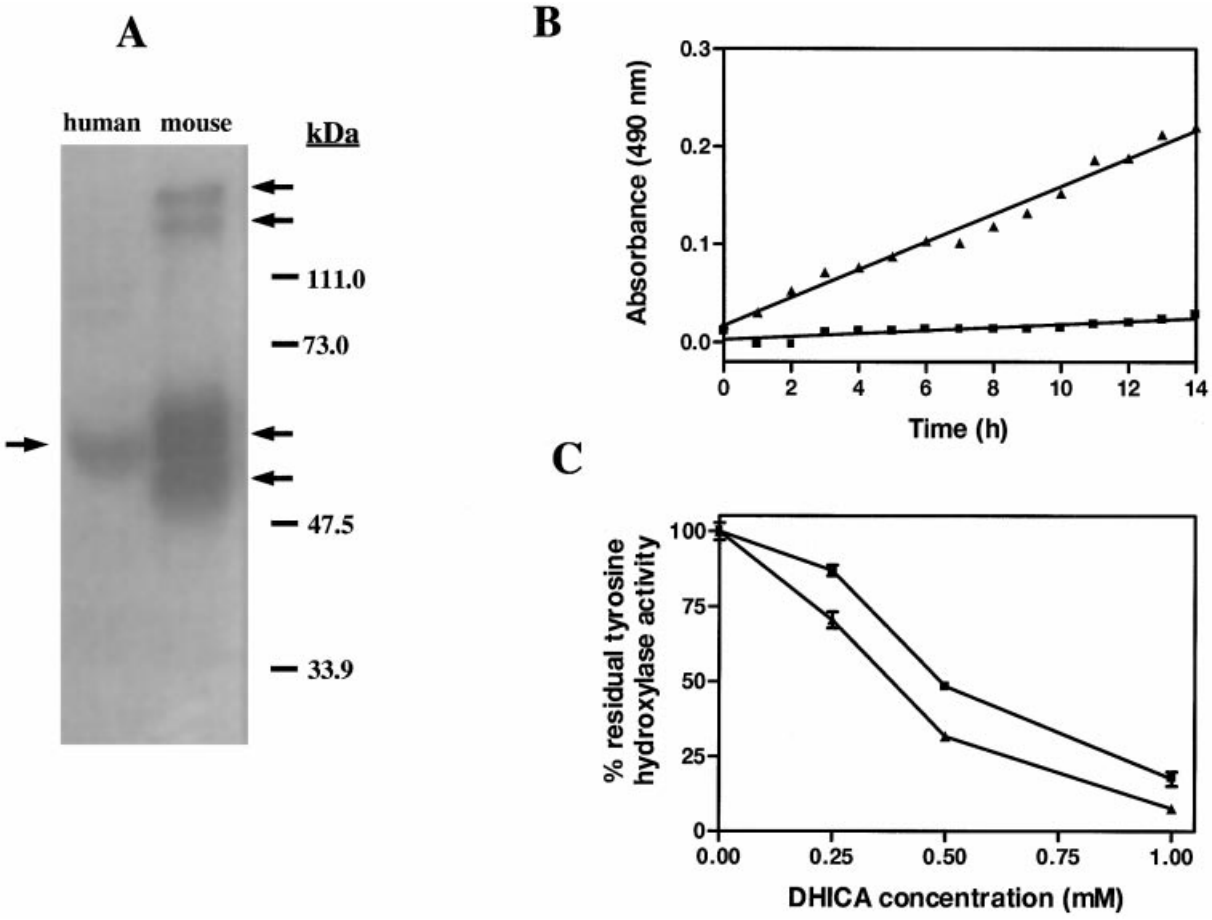

Figure 5 Recognition and oxidation of DHICA by the product of the human, but not the mouse, tyrosinase gene expressed in COS7 cells

(**A**) Efficient expression of the mouse and human tyrosinase gene in COS7 cells. Cells were harvested 24 h after transfection. An aliquot of the solubilized extract was analysed for tyrosinase expression by Western blotting, with αPEP7 as primary antibody. Molecular-mass markers are shown on the right. Arrows on the right point to the four discrete immunoreactive bands detected in cells transfected with the mouse gene, and on the left to the single band detected after transfection with the human gene. Total protein loads were 10 and 37.5 μg for cells transfected with the mouse and human genes, respectively. (**B**) Spectrophotometric demonstration of DHICA oxidation by human, but not mouse, tyrosinase. The visible spectra of 200 μM DHICA solutions in 10 mM phosphate buffer, pH 6.0/0.1 mM EDTA/2 mM MBTH, were recorded serially at 1 h intervals, in the presence of 0.6 or 6.9 m-units of dopa oxidase activity from human (▲) and mouse (■) tyrosinase transiently expressed in COS7 cells. The reference cuvette contained an identical solution, except that enzymically active tyrosinase was replaced by an equivalent amount of protein from control cells expressing the inactive Δ3tyr. The absorbance at 490 nm, corresponding to the absorption peak of the MBTH-IQCA adduct, is shown. (**C**) Inhibition of the tyrosine hydroxylase activity of human and mouse tyrosinase expressed in COS7 cells. Experimental conditions were as described for Figure 4. The extracts contained 0.4 and 2.9 μ-units of tyrosine hydroxylase activity for the human (■) and mouse (▲) enzymes, respectively. Results shown are the means $\pm$ range for two measurements.

melanins might have a higher chelating capacity, and, owing to the limitation of the number of polymerization positions available in the indole ring, they are thought to be smaller, more ordered and lighter in colour than DHI-rich pigments [41–43]. Therefore, the question of DHICA metabolism and incorporation into melanin in mammalian melanocytes is physiologically relevant.

In mouse melanocytes, DHICA is formed by the dopachrome tautomerase-catalysed tautomerization of L-dopachrome [5,9], and its incorporation into melanin is accounted for by oxidation to the corresponding and unstable quinone by tyrp1 [13,14]. Moreover, in addition to the proven ability of mouse tyrp1 to oxidize DHICA, the melanosomal protein derived from the *silver* locus could also be involved in DHICA metabolism by still uncharacterized mechanisms [37,38]. The situation is less clear in human melanocytes. In spite of extensive sequence similarity with its mouse counterpart, TYRP1 has been reported to lack DHICA oxidase activity [19], and DHICA consumption is catalysed by extracts from a human cell line completely lacking expression of TYRP1 [19]. Moreover, a DHICA-converting activity has been demonstrated for TYROSINASE, whose tyrosine hydroxylase activity is, in addition, inhibited by DHICA [22]. These data suggest that DHICA metabolism in human melanocytes can be accounted for by a TYRP1-independent, TYROSINASE-catalysed oxidation of the carboxylated dihydroxyindole. However, this hypothesis has not yet been demonstrated, since the experiments reported so far describe DHICA consumption rather than DHICA oxidation, and/or have been performed in systems where possible interference from other melanogenic proteins, such as the *silver* locus product, cannot be ruled out.

The results presented herein confirm the differences in catalytic activities of mouse and human melanogenic enzymes suggested by others [19,20], and that DHICA consumption can be catalysed by extracts from human melanocytes lacking TYRP1. Moreover, the tyrosine hydroxylase activity of TYROSINASE expressed in non-melanocytic cells is inhibited by DHICA and

the enzyme is able to catalyse DHICA consumption. In addition, we have shown that (i) DHICA consumption by human melanoma cells is accompanied by melanin formation, (ii) DHICA can partially substitute L-dopa as a cofactor of the tyrosine hydroxylase activity of TYROSINASE, irrespective of the presence or absence of TYRP1, and, more conclusively, (iii) DHICA is an authentic substrate of TYROSINASE, since it is oxidized to its quinone by human melanoma cell extracts lacking TYRP1 and by TYROSINASE expressed in COS7 cells. In this latter system, no interference by other DHICA-metabolizing melanocytic proteins, particularly the *silver* locus product [37,38], is expected. Overall, these observations prove clearly that TYROSINASE recognizes and oxidizes DHICA, thus promoting its incorporation into the melanin polymer.

The DHICA oxidase-specific activity of TYROSINASE, although easily detectable, appears low and probably smaller than the one of mouse tyrp1 [13,14]. In fact, it is within the range of the rate-limiting tyrosine hydroxylase activity, thus raising the question of its relevance in human cells. However, dopachrome tautomerase activity is also lower in human than in mouse melanocytes [44]. Thus the small DHICA oxidase activity of the human enzyme could be sufficient to oxidize DHICA under conditions of low production of the dihydroxyindole. The lower dopachrome tautomerase and DHICA oxidase activities in human cells would therefore account for the observed differences in composition of the rodent and human pigment [20,40]. However the data presented in Figures 3(B) and 5(B) show that BEU cell extracts are more efficient in catalysing the oxidation of DHICA than equivalent catalytic amounts (in terms of dopa oxidase activity) from *TYR*-transfected COS7 cells. This observation raises two possibilities. First, the catalytic efficiency towards DHICA might be lower for transiently expressed TYROSINASE than for the enzyme obtained from the melanocyte, probably as a result of a different intracellular processing. Second, melanocyte extracts might contain still uncharacterized factors catalysing DHICA oxidation, and/or co-operating with TYROSINASE to increase its DHICA oxidase activity. Both possibilities are currently being analysed in our laboratory.

A second, and related, question refers to the actual enzymic capabilities of TYRP1. Our results prove that the ability of human melanoma cell extracts to metabolize DHICA correlates approximately with their tyrosine hydroxylase and dopa oxidase activities, being the highest for SCL cells and the lowest for BEU cells. Therefore, the DHICA-converting activity of human melanocytes appears to correlate with their TYROSINASE contents. This suggests, but does not prove, that TYRP1 contributes little, if any, DHICA oxidase activity, consistent with previous reports of its lack of DHICA-converting activity [19]. Moreover, in human melanoma cell extracts, only a single activity band was detected in SDS/PAGE gels stained for dopa oxidase activity, even in highly overloaded gels. Conversely, two well-resolved bands, thought to correspond to tyrosinase and tyrp1, are usually found in mouse melanoma cells [11,13]. From the Western blots of TYRP1 (Figure 1) and TYROSINASE expressed in COS7 cells (Figure 5), the difference in molecular mass for the two human proteins appears high enough to reasonably expect their electrophoretic separation, even under the non-reducing conditions employed for activity stains. Therefore, the finding of a single activity band in human melanoma cell extracts suggests that TYRP1 lacks dopa oxidase activity, as reported by others [18]. However, other possibilities such as a low residual dopa oxidase activity or a low stability in solubilized extracts cannot be ruled out. In any case, the available evidence suggests that TYRP1 should be, at the least, less catalytically efficient than mouse tyrp1. Accordingly, its role might be more

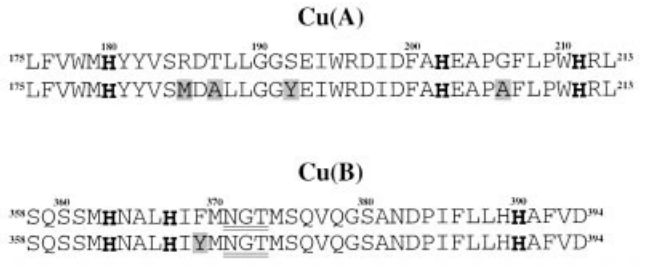

Figure 6 Amino acid sequence alignment of the copper-binding sites, Cu(A) and Cu(B), of mouse (top lines) and human (bottom lines) tyrosinases, from the first to the third essential histidines

Essential histidines in each site are shown in bold. A putative and conserved N-glycosylation site in the Cu(B) site is double underlined. Variant, non-conserved amino acids are shaded in the lower sequence, corresponding to the human enzyme.

directly related to an ability to interact with and stabilize tyrosinase [45,46]. Consistent with this, BEU cells displayed the lowest tyrosinase activity among the human melanoma cells employed in this study.

Finally, DHICA was found, surprisingly, to inhibit the tyrosine hydroxylase activity of mouse tyrosinase, in spite of the complete inability of the enzyme to catalyse its oxidation. A competitive inhibition of TYROSINASE tyrosine hydroxylase activity by DHICA was expected, based on our observation that DHICA is a substrate for the enzyme and on previous data [22]. Since DHICA binds to the mouse enzyme without being oxidized, it appears that the structural determinants responsible for ligand binding are more conserved between the human and mouse proteins than those involved in substrate transformation. The active site of tyrosinases is composed of two Cu-binding sites, designated Cu(A) and Cu(B) [8,36]. Both sites are located far apart in the primary structure of the protein, but come close enough to bind oxygen through the two copper atoms, as a result of the tridimensional folding of the molecule [47]. Interestingly, the amino acid sequence of the Cu(B) binding site is identical in the mouse and human enzymes, except for a F/Y change at position 369. However, in spite of extensive overall sequence similarity (approx. 78%), four out of 32 amino acids in the Cu(A) site are different in human and murine tyrosinase (Figure 6). The possible functional effects of these differences have not yet been assessed, but it is interesting to speculate that the structural determinants for DHICA binding and substrate recognition could be located in the highly conserved Cu(B) site. Conversely, the more divergent Cu(A) site could be involved in the different catalytic potentials of the murine and human enzyme. This possibility is being analysed by site-directed mutagenesis of selected residues located in both sites.

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REFERENCES

1 Müller, G., Ruppert, S., Schmid, E. and Schutz, G. (1988) Functional analysis of alternatively spliced tyrosinase gene transcript. EMBO J. **7**, 2723–2730
2 Kwon, B. S., Wakulchik, M., Haq, A. K., Halaban, R. and Kestler, D. (1988) Sequence analysis of mouse tyrosinase cDNA and the effect of melanotropin on its gene expression. Biochem. Biophys. Res. Commun. **153**, 1301–1309

3 Shibahara, S., Tomita, Y., Sakakura, T., Nager, C., Chaudhuri, B. and Muller, R. (1986) Cloning and expression of cDNA encoding mouse tyrosinase. Nucleic Acids Res. **14**, 2413–2427

4 Jackson, I. J. (1988) A cDNA encoding tyrosinase-related protein maps to the brown locus in mice. Proc. Natl. Acad. Sci. U.S.A. **85**, 4392–4396

5 Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. M. and Hearing, V. J. (1992) A second tyrosinase related protein (TRP-2) is a melanogenic enzyme termed dopachrome tautomerase. EMBO J. **11**, 519–526

6 Jackson, I. J., Chambers, D. M., Tsukamoto, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Hearing, V. J. (1992) A second tyrosinase related protein, TRP-2 maps to and is mutated at the mouse slaty locus. EMBO J. **11**, 527–535

7 Cooksey, C., Garrat, P., Land, E., Pavel, S., Ramsden, C., Riley, P. and Smit, N. (1997) Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. J. Biol. Chem. **272**, 26226–26235

8 Prota, G. (1992) An introduction to melanin research. In Melanins and Melanogenesis, pp. 1–290, Academic Press, New York

9 Aroca, P., García-Borrón, J. C., Solano, F. and Lozano, J. A. (1990) Regulation of distal mammalian melanogenesis. I. Partial purification and characterization of a dopachrome converting factor: dopachrome tautomerase. Biochim. Biophys. Acta **1035**, 266–275

10 Palumbo, A., Solano, F., Misuraca, G., Aroca, P., García.Borrón, J. C., Lozano, J. A. and Prota, G. (1991) Comparative actions of dopachrome tautomerase and metal ions on the rearrangement of dopachrome. Biochim. Biophys Acta **1115**, 1–5

11 Jiménez-Cervantes, C., García-Borrón, J. C., Valverde, P., Solano, F. and Lozano, J. A. (1993) Tyrosinase isoenzymes in mammalian melanocytes. I. Biochemical characterization of two melanosomal tyrosinases from B16 mouse melanoma. Eur. J. Biochem. **217**, 549–556

12 Jiménez, M., Tsukamoto, K. and Hearing, V. (1991) Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. J. Biol. Chem. **266**, 1147–1156

13 Jiménez-Cervantes, C., Solano, F., Kobayashi, T., Urabe, K., Hearing, V. J., Lozano, J. A. and García-Borrón, J. C. (1994) A new enzymatic function in the melanogenic pathway: the DHICA oxidase activity of tyrosinase related protein-1 (TRP1). J. Biol. Chem. **269**, 17993–18001

14 Kobayashi, T., Urabe, K., Winder, A., Jiménez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., García-Borrón, J. C. and Hearing, V. (1994) Tyrosinase-related protein 1 (TRP1) functions as a DHICA oxidase in melanin byosinthesis. EMBO J. **13**, 5818–5825

15 Nishioka, K. (1977) Conversion of particulate tyrosinase to soluble form and to desialytated tyrosinase in human malignant melanoma. FEBS Lett. **80**, 225–229

16 Jergil, B., Lindbladh, C., Rorsman, H. and Rosengren, E. (1983) Dopa oxidation and tyrosine oxygenation by human melanoma tyrosinase. Acta Derm. Venereol. (Stockholm) **63**, 468–475

17 Laskin, J. D. and Piccinini, L. (1986) Tyrosinase isozyme heterogeneity in differentiating B16/c3 melanoma. J. Biol. Chem. **261**, 16626–16635

18 Zhao, H., Zhao, Y., Nordlund, J. and Boissy, R. (1994) Human TRP-1 has tyrosine hydroxylase but no dopa oxidase activity. Pigment Cell Res. **7**, 131–140

19 Boissy, R., Sakai, C., Zhao, H., Kobayashi, T. and Hearing, V. (1998) Human tyrosinase related protein-1 (TYRP1) does not function as a DHICA oxidase activity in contrast to murine TYRP1. Exp. Dermatol. **7**, 198–204

20 Wilczek, A., Kondoh, H. and Mishima, Y. (1996) Composition of mammalian eumelanins: analyses of DHICA-derived units in pigments from hair and melanoma cells. Pigment Cell Res. **9**, 63–67

21 Hunt, G., Kyne, S., Ito, S., Wakamatsu, K., Todd, C. and Thody, A. J. (1995) Eumelanin and phaeomelanin contents of human epidermis and cultured melanocytes. Pigment Cell Res. **8**, 202–208

22 Wilczek, A. and Mishima, Y. (1995) Inhibitory effects of melanin monomers, dihydroxyindole-2-carboxylic acid (DHICA) and dihydroxyindole (DHI) on mammalian tyrosinase, with a special reference to the role of DHICA/DHI ratio in melanogenesis. Pigment Cell Res. **8**, 105–112

23 Del Marmol, V., Ito, S., Jackson, I. J., Vachtenheim, J., Berr, P., Ghanem, G., Morandini, R., Wakamatsu, K. and Huez, G. (1993) TRP-1 expression correlates with eumelanogenesis in human pigment cells in culture. FEBS Lett. **327**, 307–310

24 Bouchard, B., del Marmol, V., Jackson, I. J., Cherif, D. and Dubertret, L. (1994) Molecular characterization of a human tyrosinase-related-protein-2 cDNA. Patterns of expression in melanocytic cells. Eur. J. Biochem. **219**, 127–134

25 Eberle, J., Garbe, C., Wang, N. and Orfanos, C. (1995) Incomplete expression of the tyrosinase gene family (tyrosinase, TYRP1 and TYRP-2) in human malignant melanoma cells *in vitro*. Pigment Cell Res. **8**, 307–313

26 Martínez-Esparza, M., Jiménez-Cervantes, C., Beermann, F., Aparicio, P., Lozano, J. A. and García-Borrón, J. C. (1997) Transforming growth factor-β1 inhibits basal melanogenesis in B16/F10 mouse melanoma cells by increasing the rate of degradation of tyrosinase and tyrosinase-related protein-1. J. Biol. Chem. **272**, 3967–3972

27 Jara, J. R., Solano, F. and Lozano, J. A. (1988) Assays for mammalian tyrosinase: a comparative study. Pigment Cell Res. **1**, 332–339

28 Winder, A. J. and Harris, H. (1991) New assays for tyrosine hydroxylase and dopa oxidase activities of tyrosinase. Eur. J. Biochem. **198**, 317–326

29 Jiménez-Cervantes, C., Valverde, P., García-Borrón, J. C., Solano, F. and Lozano, J. A. (1993) Improved tyrosinase activity stains in polyacrylamide electrophoresis gels. Pigment Cell Res. **6**, 394–399

30 Loir, B., Pérez Sánchez, C., Ghanem, G., Lozano, J. A., García-Borrón, J. C. and Jiménez-Cervantes, C. (1999) Expression of the MC1 receptor gene in normal and malignant human melanocytes. A semiquantitative RT-PCR study. Cell. Mol. Biol. **45**, 1083–1092

31 Yamamoto, H., Takeuchi, S., Kudo, T., Makino, K., Nakata, A., Shinoda, T. and Takeuchi, T. (1987) Cloning and sequencing of mouse tyrosinase cDNA. Jpn. J. Genet. **62**, 271–274

32 Kelsall, S. R., Le Fur, N. and Mintz, B. (1997) Qualitative and quantitative catalog of tyrosinase alternative transcripts in normal murine skin melanocytes as a basis for detecting melanoma-specific changes. Biochem. Biophys. Res. Commun. **236**, 173–177

33 Kwon, B., Haq, A., Pomerantz, S. and Halaban, R. (1987) Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. Proc. Natl. Acad. Sci. U.S.A. **84**, 7473–7477

34 Giebel, L. B. and Spritz, R. A. (1990) RFLP for Mbo I in the human tyrosinase (TYROSINASE) gene detected by PCR. Nucleic Acids Res. **18**, 3103

35 Bu'Lock, J. D. (1960) Intermediates of melanin formation. Arch. Biochem. Biophys. **91**, 189–193

36 Riley, P. (1999) The great DOPA mistery: the source and significance of DOPA in phase I melanogenesis. Cell. Mol. Biol. **45**, 951–960

37 Chakraborty, A. K., Platt, J., Kim, K., Kwon, B., Bennett, D. and Pawelek, J. M. (1996) Polymerization of 5,6-dihydroxyindole-2-carboxylic acid to melanin by the pmel17/silver locus protein. Eur. J. Biochem. **236**, 180–188

38 Lee, Z., Hou, L., Moellmann, G., Kublinska, E., Antol, K., Fraser, M., Halaban, R. and Kwon, B. (1996) Characterization and subcellular localization of human Pmel17/silver, a 100 kDa (pre)melanosomal membrane protein associated with 5,6-dihydroxyindole-2-carboxylic acid (DHICA) converting activity. J. Invest. Dermatol. **106**, 605–610

39 Ito, S. and Wakamatsu, K. (1989) Melanin chemistry and melanin precursors in melanoma. J. Invest. Dermatol. **92**, 261S–265S

40 Ozeki, H., Ito, S., Kakamatsu, K. and Hirobe, T. (1995) Chemical characterization of hair melanins in various coat-color mutants of mice. J. Invest. Dematol. **105**, 361–366

41 Palumbo, A., d'Ischia, M. and Prota, G. (1987) Tyrosinase-promoted oxidation of 5,6-dihydroxyindole-2-carboxylic acid to melanin. Isolation and characterization of oligomer intermediates. Tetrahedron **43**, 4203–4206

42 Aroca, P., Solano, F., Salinas, C., García-Borrón, J. C. and Lozano, J. A. (1992) Regulation of the final phase of mammalian melanogenesis. The role of dopachrome tautomerase and the ratio between 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole. Eur. J. Biochem. **208**, 155–163

43 Orlow, S., Osber, M. and Pawelek, J. (1992) Synthesis and characterization of melanins from dihydroxyindole-2-carboxylic acid and dihydroxyindole. Pigment Cell Res. **5**, 113–121

44 Bernd, A., Ramirez-Bosca, A., Kippenberger, S., Martinez Liarte, J. H., Holzmann, H. and Solano, F. (1994) Levels of dopachrome tautomerase in human melanocytes cultured *in vitro*. Melanoma Res. **4**, 287–291

45 Jiménez-Cervantes, C., Martínez-Esparza, M., Solano, F., Lozano, J. A. and García-Borrón, J. C. (1998) Molecular interactions within the melanogenic complex: formation of heterodimers of tyrosinase and TRP1 from B16 mouse melanoma. Biochem. Biophys. Res. Commun. **253**, 761–767

46 Kobayashi, T., Imokawa, G., Bennett, D. and Hearing, V. J. (1998) Tyrosinase stabilization by tyrp1 (the brown locus protein). J. Biol. Chem. **273**, 31801–31805

47 Lowery, M. D. and Solomon, E. I (1993) Electronic structure contributions to function in bioinorganic chemistry. Science **259**, 1575–1581

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