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Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis

Takeshi Kobayashi, Kazunori Urabe, Alison Winder[1,2], Celia Jiménez-Cervantes[3], Genji Imokawa[4], Timothy Brewington, Francisco Solano[3], José Carlos García-Borrón[3] and Vincent J.Hearing[5]

Laboratory of Cell Biology, National Cancer Institute, Building 37, Room 1B22, National Institutes of Health, Bethesda, MD 20892 USA, [1]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK, [3]Department of Biochemistry, Faculty of Medicine, University of Murcia, Murcia, Spain, and [4]Kao Institute for Fundamental Research, Akabane, Ichikaimachi, Haga, Tochigi 321–34 Japan
[2]Current address: Glaxo Group Research Limited, Molecular Sciences, Greenford Road, Greenford, Middlesex UB6 0HE, UK

[5]Corresponding author

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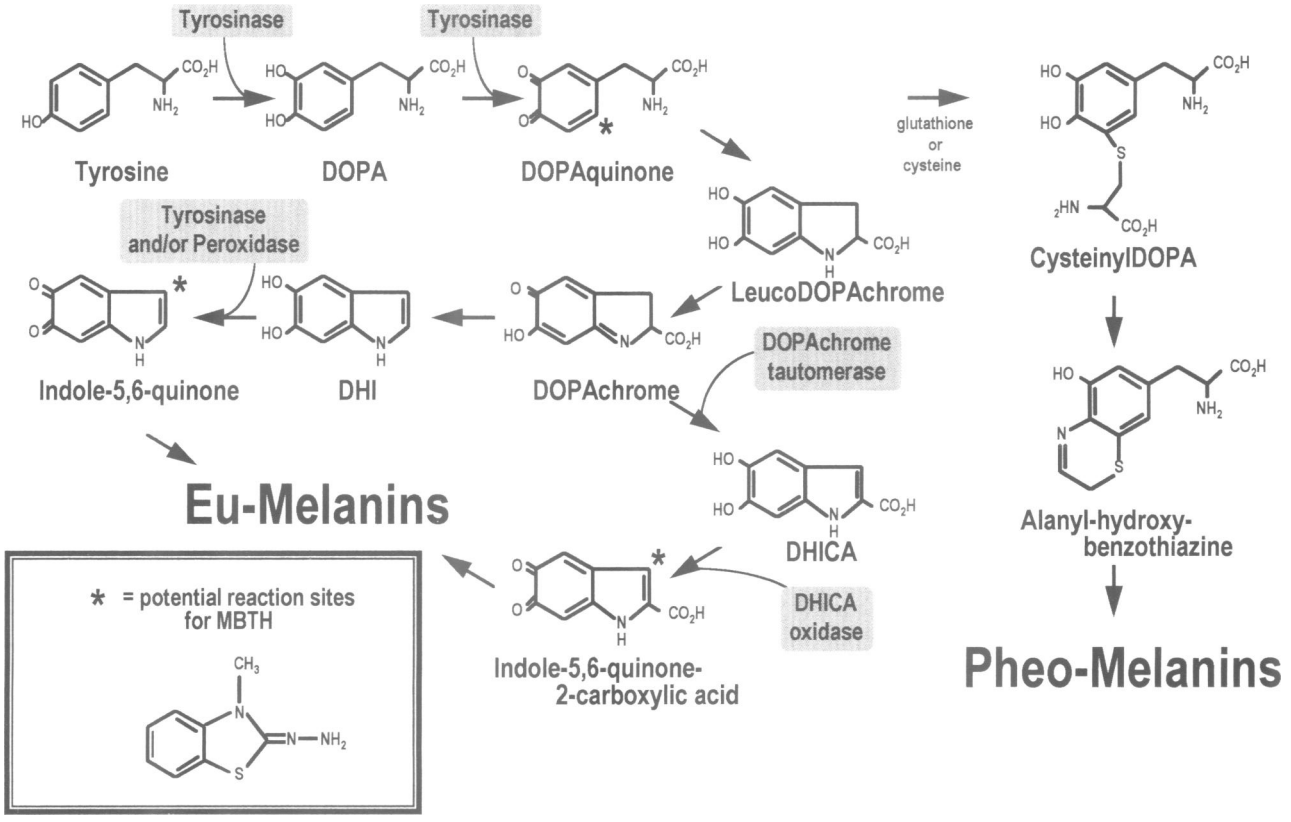
Several genes critical to the enzymatic regulation of melanin production in mammals have recently been cloned and mapped to the *albino*, *brown* and *slaty* loci in mice. All three genes encode proteins with similar structures and features, but with distinct catalytic capacities; the functions of two of those gene products have previously been identified. The *albino* locus encodes tyrosinase, an enzyme with three distinct melanogenic functions, while the *slaty* locus encodes tyrosinase-related protein 2 (TRP2), an enzyme with a single specific, but distinct, function as DOPAchrome tautomerase. Although the *brown* locus, encoding TRP1, was actually the first member of the tyrosinase gene family to be cloned, its catalytic function (which results in the production of black rather than brown melanin) has been in general dispute. In this study we have used two different techniques (expression of TRP1 in transfected fibroblasts and immunoaffinity purification of TRP1 from melanocytes) to examine the enzymatic function(s) of TRP1. The data demonstrate that the specific melanogenic function of TRP1 is the oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to a carboxylated indole-quinone at a downstream point in the melanin biosynthetic pathway. This enzyme activity appears to be essential to the further metabolism of DHICA to a high molecular weight pigmented biopolymer.

Key words: melanin/melanogenesis/pigmentation/TRP1/tyrosinase

Introduction

Recent advances using techniques of molecular biology and biochemistry have demonstrated that the chemical pathway for melanin biosynthesis in mammalian melanocytes is much more complex than was originally thought. From an enzymatic point of view, tyrosinase [EC 1.14.18.1] is the only catalyst absolutely required to initiate the cascade of reactions necessary to convert the amino acid tyrosine to the melanin biopolymer (Körner and Pawelek, 1982; Hearing, 1987; see Scheme 1). However, it is now known that a number of other melanogenic factors participate in modulating those reactions (reviewed by Hearing and Tsukamoto, 1991; Pawelek, 1991), and one example of such a post-tyrosinase factor is another melanogenic enzyme, DOPAchrome tautomerase [EC 5.3.2.3]. This latter enzyme diverts the spontaneous decarboxylative conversion of DOPAchrome to 5,6-dihydroxyindole (DHI) instead of to the carboxylated derivative termed DHI-2-carboxylic acid (DHICA; Körner and Pawelek, 1980; Barber *et al.*, 1984; Aroca *et al.*, 1990; Pawelek, 1990). It has been recently demonstrated that DHICA and/or DHICA derivatives are incorporated into melanins to a significant extent *in vivo* (Ito and Wakamatsu, 1989; Tsukamoto *et al.*, 1992c) although it is not yet clear exactly what effect this might have on the structure or function of the melanins produced. DHICA has been shown to be a relatively stable intermediate which is a major component in coated vesicles in transit to melanosomes (Hatta *et al.*, 1988; Mishima, 1992; Shibata *et al.*, 1993). The manner in which the DHICA is subsequently metabolized to the melanin biopolymer upon delivery to the melanosomes is currently unknown, but has been postulated to be controlled by an unknown post-tyrosinase factor (Wilczek and Mishima, 1993) and has been implicated with the catalytic function of a melanogenic enzyme, low electrophoretic mobility tyrosinase (LEMT; Jiménez-Cervantes *et al.*, 1994).

During the elaboration of this biochemical pathway, remarkable progress has concurrently been made in the elucidation of the genetic influences responsible for regulating mammalian pigmentation. It has been known for some time that >60 different genetic loci regulate melanogenesis in mice at various levels: more than a dozen of these genes have now been cloned, and in some cases, their mechanism of interaction with respect to melanogenesis has been characterized even in humans (reviewed by Hearing and Tsukamoto, 1991; Hearing, 1993; Urabe *et al.*, 1993). Several of those genes are expressed specifically by melanocytes, and exert their function at the level of melanin biosynthesis and melanosomal biogenesis. Some of those cloned genes, e.g. *silver* (Kwon *et al.*, 1987; 1991) and *pink-eyed dilution* (Gardner *et al.*, 1992; Brilliant, 1992; Rinchik *et al.*, 1993) encode proteins that are apparently involved in the structure or function of melanosomes at the organelle level while others, such as *albino* and *slaty*, encode proteins that function specifically at the level of enzymatic activity in melanogenesis. Many different studies have shown that



Scheme 1. Melanogenic pathway. The series of chemical reactions involved in the production of eumelanins and pheomelanins are shown, as are the regulatory enzymes involved. Inset: structure of MBTH; quinones are trapped by reaction with the hydrazone group at the potential sites in the scheme indicated by an asterisk.

the *albino* locus encodes tyrosinase, the trifunctional and rate-limiting melanogenic enzyme noted above. Mutations at the *albino* locus elicit dramatic effects on the quantity of melanin produced, and typically little or no melanin is produced in mutant animals (King *et al.*, 1994). The *slaty* locus encodes DOPAchrome tautomerase (Jackson, 1988; Jackson *et al.*, 1992; Tsukamoto *et al.*, 1992a), an enzyme with a high degree of homology to tyrosinase, but which has a specific and distinct function in melanin biosynthesis, also as noted above.

Although the *brown* locus was chronologically the first member of the tyrosinase gene family to be cloned (Shibahara *et al.*, 1986; Jackson, 1988) it has remained the most resistant to definition of a specific melanogenic function. TRP1 has been proposed to act as another, lower specific activity, tyrosinase (Jiménez *et al.*, 1989, 1991; Jiménez-Cervantes *et al.*, 1994), another DOPAchrome tautomerase (Jackson, 1988; Winder *et al.*, 1993b), or a melanocyte specific catalase (Halaban and Moellmann, 1990). For various reasons, none of those functions has been unequivocally accepted. Whatever its enzyme function, we can surmise that the function of TRP1 controls the quality of melanin produced, since mutations in its structural gene elicit the production of a lighter colored brown melanin in the hair of mutant animals (e.g. brown, *b/b* or brown cordovan, b^c/b^c) than that seen in mice wild-type at the *brown* locus. By comparison, a point mutation in the structural locus for TRP2 (slaty, *slt*) results in a significant reduction of DOPAchrome tautomerase activity in epidermal and ocular melanocytes and the lessening of dark black melanin production in the hair of mutant animals to a dark grey color (Jackson *et al.*, 1992).

Tyrosinase can function distally in the melanogenic pathway as a DHI oxidase (Körner and Pawelek, 1982; Hearing *et al.*, 1982; Tripathi *et al.*, 1992) and it has been proposed that peroxidase might also promote the polymerization of DHI and DHICA (d'Ischia *et al.*, 1990). However, melanosomes have virtually negligible levels of peroxidase activity (Tsukamoto *et al.*, 1992b). When murine melanoma cells were stimulated with α-melanocyte stimulating hormone (MSH), tyrosinase activity and expression was drastically increased while DHICA oxidase and DOPAchrome tautomerase activities were constant, as was TRP1 and TRP2 expression (Aroca *et al.*, 1993). Taken together, these lines of evidence suggest that some enzyme other than tyrosinase or peroxidase regulates DHICA metabolism in melanocytes, possibly TRP1 and/or TRP2. Finally, genetic evidence suggests that TRP1 functions at or later in the melanogenic pathway than does TRP2/DOPAchrome tautomerase (I.Jackson, personal communication).

As noted above, we have recently obtained evidence that a biochemically purified tyrosinase-like enzyme, LEMT, reacted specifically with an antibody against TRP1 and showed a novel DHICA oxidase activity (Jiménez-Cervantes *et al.*, 1994). In this study therefore, we have examined the potential use by TRP1 of metabolites in the melanogenic pathway downstream of the action of TRP2 (i.e. DHI and DHICA). We used two different approaches (transfection of genes encoding TRP1 and/or tyrosinase

Table I. DHI and DHICA oxidase activities of murine melanocytes

Cell line (genotype)	Substrate and assay method		
	DHI (HPLC)	DHICA (HPLC)	DHICA (MBTH)
Melan-a (black)	608 ± 225 (4)	453 ± 107 (5)	81 ± 11 (3)
Melan-b (brown)	46% ± 15%[a]	70% ± 9%[a]	49% ± 16%[a]
Melan-c (albino)	0% ± 0%[a]	105% ± 10%	102% ± 49%

The ability of extracts of immortalized melanocytes to use DHI or DHICA as substrates was assayed using HPLC and/or MBTH, as detailed in Materials and methods. Results are presented as mean ± SEM (n). Units for melan-a are pmol/μg protein/h. Results for melan-b and melan-c are % activity compared with the melan-a controls (n = same as melan-a). Backgrounds have been subtracted and were established using extracts of NIH 3T3 cells as negative controls.
[a]$P < 0.01$ from melan-a as a control.

Table II. Abilities of transfected cell extracts to use DHI and DHICA as substrates

Cell line	Transfected with	Substrate and assay method	
		DHI (HPLC)	DHICA (HPLC)
Clone c	tyrosinase only	22 ± 10 (3)	0 ± 0 (5)
Clone b	TRP1 only	68 ± 55 (3)	151 ± 38 (5)[a]
Clone c+b	tyrosinase + TRP1	138 ± 51 (3)[a]	198 ± 75 (4)*

The abilities of extracts of transfected fibroblasts to oxidize DHI and/or DHICA were measured by HPLC, as detailed in Materials and methods section. Results are presented as means ± SEM (n) in units of pmol/μg protein/h; backgrounds have been subtracted and were established using extracts of untransfected 3T3 Swiss and neomycin-only transfected 3T3 Swiss cells as negative controls.
[a]$P < 0.01$ from clone c as a control.

into fibroblasts and immunoaffinity purification of those two melanogenic enzymes) to examine the enzymatic function(s) of TRP1, and we now present evidence that the specific function of TRP1 in the melanogenic pathway is as a DHICA oxidase.

Results

DHI and DHICA conversion activity of melanocyte extracts

As noted above, genetic and immunological evidence suggests that TRP1 functions distally to TRP2 (DOPA-chrome tautomerase) in melanogenesis, quite possibly therefore at the level of DHICA and/or DHI metabolism (Scheme 1). In an initial screening to evaluate the potential catalytic function of TRP1 towards DHI and DHICA, we characterized the ability of extracts of genetically diverse melanocytes to use those melanogenic intermediates as substrates to generate indole-quinones. The data in Table I show that the ability of melan-b (brown, i.e. mutant TRP1) cells to metabolize DHI or DHICA is significantly reduced compared with the activity of melan-a (black, i.e. wild-type cells). Generation of an indole-quinone product, assessed using the 3-methyl-2-benzothiazolinone hydrazone (MBTH) assay (see below) confirmed that melan-b cells have significantly less DHICA conversion activity than melan-a and melan-c (albino) cells, supporting the concept that TRP1, but not tyrosinase, may be a critical enzyme in DHICA metabolism. Note that while DHICA oxidation by melan-c (albino) cells was equivalent to that of melan-a cells, melan-c cells had no ability to oxidize DHI, suggesting that tyrosinase (mutant in those cells) contains all of that activity.

Expression and catalytic function of melanogenic proteins in transfected fibroblasts

One approach to definitively characterizing the function of a gene product is to express that gene in a cell type in which it normally is not expressed. We have transfected genes encoding tyrosinase and/or TRP1 into nonmelanocytes (i.e. fibroblasts) for this purpose. Examination of the expression of tyrosinase and TRP1 by transfected fibroblasts using metabolic labeling and immunoprecipitation with specific antibodies (Figure 1) demonstrated that the expression of those proteins is as would be predicted. Clone c, transfected with tyrosinase, expresses only tyrosinase; clone b, transfected only with TRP1, expresses only TRP1, while clone c+b was transfected with both tyrosinase and TRP1 and both those proteins are expressed. Neither tyrosinase nor TRP1 was expressed by the original fibroblasts (f) used for the transfection or by fibroblasts transfected with the selectable marker only (neo). Western blot analysis of cell extracts revealed a similar pattern of expression of these transfected proteins (data not shown).

When we measured the enzymatic activities of extracts of those transfected cells, both cell lines transfected with and expressing TRP1 (i.e. clone b and clone c+b) had relatively high catalytic function towards DHICA as a substrate although the transfected cell line expressing tyrosinase only (i.e. clone c) had no such ability (Table II). All three transfected cell lines appeared able to use DHI as a substrate, with the highest activity shown by clone c+b.

Purity and catalytic function of immunoaffinity purified proteins

In a second approach to defining the putative melanogenic function(s) of TRP1 in DHI and DHICA metabolism, we have used specific antibodies attached to a solid phase matrix to purify tyrosinase and TRP1 from extracts of melanoma cells. Western immunoblots (Figure 2) demonstrate the purity of the tyrosinase and TRP1 used in these studies. Each filter contains TRP1 (purified from wild-type black B16 cells), mutant TRP1 (purified from brown mutation, b/b, S91 cells), and tyrosinase (purified from wild-type B16 cells). When the filter was incubated with the antibody against TRP1 (αPEP1), only bands in the purified TRP1 fractions were recognized. A minor low molecular weight band in the wild-type TRP1 represents the *de novo* form of the enzyme while the major higher molecular weight band represents the glycosylated form, predominant in these cells. Note that the mutant TRP1 has an apparent molecular weight lower than that of the wild-type TRP1, which may reflect the effect of the point mutation in the former. When identical blots were incubated with the antibody recognizing tyrosinase (αPEP7), only the purified tyrosinase was reactive with that antibody. Again, the lower molecular weight form represents the *de novo* synthesized form of tyrosinase while the higher molecular weight form represents the predominant glycosylated form. The differences in

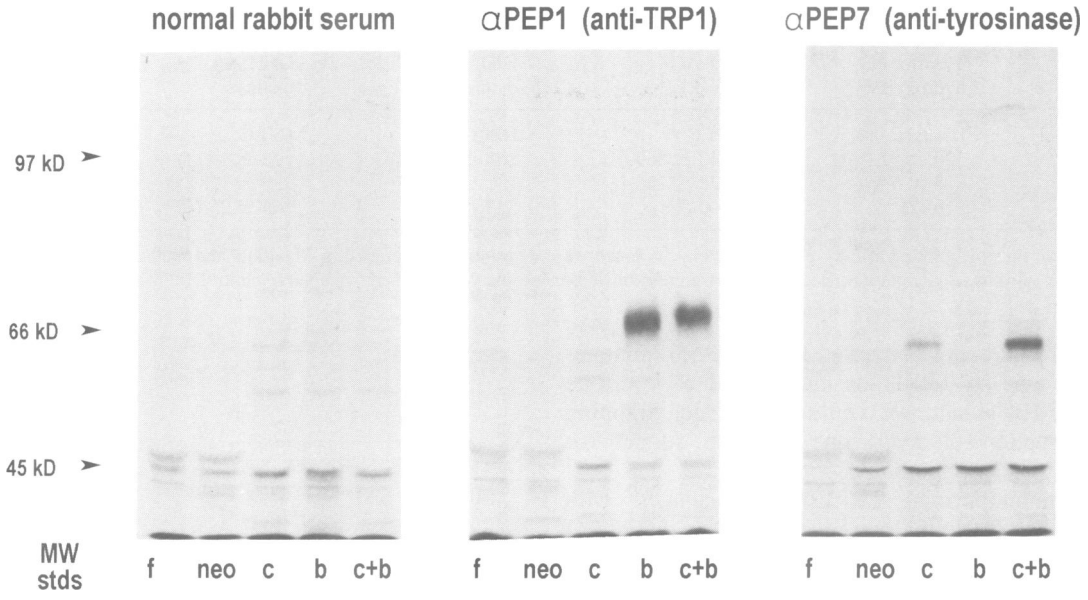


Fig. 1. Production of TRP1 and/or tyrosinase by transfected cells. Transfected 3T3 Swiss fibroblasts were metabolically labeled, immunoprecipitated with peptide specific antisera and visualized by autoradiography, as described in Materials and methods. f = control (untransfected); neo = neomycin resistance gene transfected only; c = clone c (tyrosinase only); b = clone b (TRP1 only); c + b = clone c+b (tyrosinase and TRP1).

glycosylation processing and patterns of tyrosinase and TRP1 have been previously described by our group (Tsukamoto *et al.*, 1992b). As can be seen from this figure, there is no detectable cross-contamination of immunoaffinity purified tyrosinase or TRP1.

Analyses of the abilities of those same immune-purified enzymes to use various melanogenic precursors and intermediates as substrates (Table III) demonstrated that, consistent with the results on transfected cells presented above, tyrosinase can utilize DHI relatively well as a substrate (and DHICA only very poorly), whereas TRP1 has a relatively higher activity towards DHICA as a substrate. Although it appears from the HPLC assay results (Tables II and III) that TRP1 might oxidize DHI, that activity is not statistically significant above background and at this time must be considered tentative at best. Due to the unstable nature of DHI and, to a lesser degree DHICA, it is relatively difficult to establish accurately exact backgrounds in these oxidation experiments; in addition, minor changes in assay conditions, such as in pH or the presence of endogenous reducing compounds, can significantly affect rates of spontaneous oxidation. The MBTH assay circumvents some of those limitations and when used to assay these same fractions confirms that tyrosinase is 10 times less efficient than TRP1 in its ability to use DHICA as a substrate (a result corroborating the transfection studies presented above). Although TRP1 isolated from brown mutant S91 cells (i.e. TRP1 b/b) has a slightly diminished activity towards DHICA compared to wild-type TRP1 using the HPLC assay, this difference is not statistically significant and is not reflected in the MBTH assay results.

Metabolism of DHICA by TRP1

Our results using two different approaches demonstrated that TRP1 has the ability to use DHICA and, to a lesser extent, DHI as substrates. An important question then

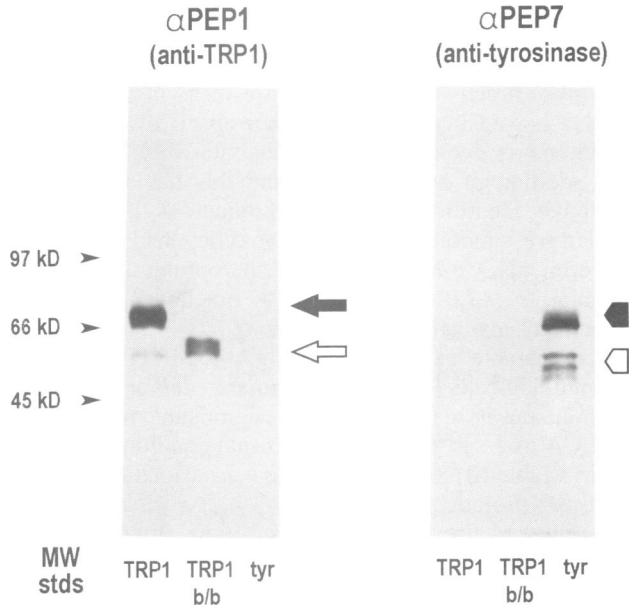


Fig. 2. Western immunoblotting of immunoaffinity purified proteins. Tyrosinase (tyr) and TRP1 from B16 cells, and TRP1 from S91 cells (TRP1 b/b), were immunoaffinity purified and reacted by Western blotting with the antibodies noted. Visualization of specifically bound antibodies was by enhanced chemiluminescence.

remains as to the products generated by those reactions. As an avenue to identifying the product of the catalytic action of TRP1 on DHICA, we used the MBTH assay originally described by Winder and Harris (1991) for DOPA oxidase; this assay has been subsequently modified to measure DHI and DHICA oxidase activities (Jiménez-Cervantes *et al.*, 1994). MBTH captures the indole–quinone generated by the oxidation of dihydroxyindole compounds (Scheme 1 inset), and it would be expected

Table III. Abilities of affinity-purified proteins to use melanogenic substrates

	Tyrosine hydroxylase	DOPA oxidase	DHI oxidase	DHICA oxidase	DHICA oxidase
Assay method	[^{3}H]tyrosine	HPLC	HPLC	HPLC	MBTH
Tyrosinase	325 ± 57 (5)	3542 ± 869 (4)	863 ± 209 (4)	25 ± 14 (4)	24 ± 21 (3)
TRP1	8 ± 5 (5)	28 ± 23 (4)	131 ± 269 (5)	239 ± 128 (5)	310 ± 41 (3)
TRP1 b/b	6 (2)	0 (1)	353 (2)	66 ± 25 (6)	424 (1)

Melanogenic activities of immune-affinity purified proteins as noted were determined as detailed in Materials and methods and are reported as mean ± SEM (*n*) in pmol/μg protein/h. Backgrounds were established using dialysis buffer as a negative control.

that if DHICA is being converted to indole-5,6-quinone-2-carboxylic acid by TRP1, the indole product would be trapped by MBTH and we could detect such trapping as the production of hydrazone−quinone adduct (or conjugate) by spectrophotometry.

When immunopurified TRP1 was incubated with DHICA and MBTH in the presence of EDTA, the rate of production of an absorbing species (absorbance peak at 470 nm) was significantly increased above the background rate of auto-oxidation (Figure 3). Judging from the linear slope, it is clear that TRP1 can catalyze DHICA in a reaction in which the product can be trapped by MBTH; the product is tentatively assumed to be the indole−quinone derivative as noted above. A related study (Jiménez-Cervantes *et al.*, 1994) has demonstrated the ability of a biochemically purified fraction, LEMT, to use DHICA as a substrate; although the product of the reaction has not yet been directly identified because of its instability, that study showed that it can also be trapped by MBTH. In addition, its evolution can be quantitatively delayed by the addition of ascorbic acid, and this has allowed the tentative identification of the product as the quinone derivative, indolequinone-2-carboxylic acid. In other experiments, we have found that the optimum pH of this reaction is ~7.0 and that TRP1 functions in a dose-dependent manner (data not shown).

Comparable experiments using the MBTH assay on immunologically purified tyrosinase demonstrate that mammalian tyrosinase has no significant function on DHICA as a substrate under the usual conditions of a 1 h assay (Table III). However, we have occasionally observed that with prolonged incubation (>1 h), tyrosinase displays an ability to use DHICA as a substrate (Figure 3); this 'lag' period is similar to that noted with the tyrosine hydroxylase activity of tyrosinase although its significance is unknown.

Expression of melanogenic activities in follicular melanocytes

An examination of the melanogenic activities in whole skin or hairbulb preparations from 10-day-old mice is consistent with the DHICA oxidase function of TRP1 (Table IV). Cordovan is a mutation at the brown locus in which TRP1 expression is minimal (<1% of the wild-type; Jackson, 1994) similar to that described for Cordovan-Harwell (Jackson *et al.*, 1990). Although levels of tyrosinase and DOPAchrome tautomerase activities in extracts of cordovan skin are similar to those present in extracts of black skin, the level of DHICA oxidase activity is at or below background (top of Table IV). The expression of melanogenic proteins in pigment mutations (such as

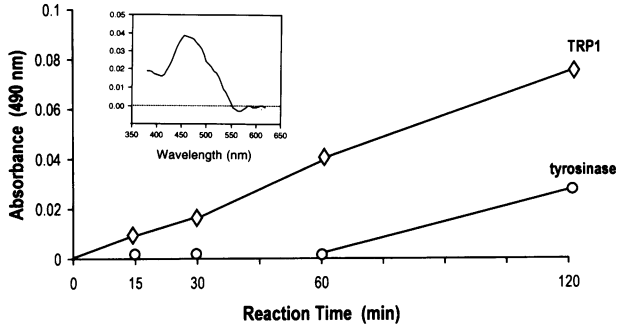


Fig. 3. Capture of DHICA reaction product by MBTH. DHICA was reacted at 37°C with buffer, or with immunoaffinity purified TRP1 or tyrosinase in the presence of MBTH and EDTA. The results are presented as the means ± SEM of the reactions over 2 h. Background established by auto-oxidation has been subtracted from the data. Inset: absorbance curve of TRP1/DHICA sample at 60 min.

lethal yellow), which produce pheomelanin rather than eumelanin, is significantly reduced compared with that in black melanocytes, and expression of TRP1 and TRP2 is completely absent (Kobayashi *et al.*, in preparation). As can be seen in the bottom of Table IV, the DOPAchrome tautomerase and DHICA oxidase activities of lethal yellow hair bulbs are at background levels, consistent with the absence of TRP2 and TRP1, respectively.

Discussion

In this study we have shown that TRP1 has the ability to use DHICA as a substrate for oxidation; this catalytic function has been found for TRP1 immunopurified from extracts of melanoma cells and for TRP1 expressed in transfected fibroblasts. The data are less conclusive about whether TRP1 may also be able to use DHI as a substrate to a minor extent. Conversely, tyrosinase is able to use DHI as a substrate but uses DHICA very poorly, if at all, and thus we suggest that the specific role that TRP1 plays in eumelanogenesis is at the distal step in the pathway as a DHICA oxidase. This finding is consistent with earlier predictions by Jackson based on genetic evidence that the catalytic function of TRP2 (DOPAchrome tautomerase) preceeds that of TRP1, and is also consistent with recent findings that TRP1 expression is exclusively associated with the eumelanic pathway (Del-Marmol *et al.*, 1993), a notion initially suggested by Houghton *et al.* (1987).

Mice homozygous for the recessive *brown* mutation are brown rather than black, but the chemical and/or physical difference(s) responsible for this phenotype is not known. Our data suggest that altered DHICA oxidase metabolism may in part be responsible for this visible difference; we

Table IV. Melanogenic activities in murine melanocytes

	Tyrosine hydroxylase	DOPA oxidase	DOPAchrome tautomerase	DHI oxidase	DHICA oxidase	Melanin formation
Whole skin (from 10-day-old mice)						
black (n = 5)	18 ± 3	194 ± 35	12 ± 5	18 ± 3	5 ± 1	5 ± 1
brown (n = 3)	15 ± 3	162 ± 27	4 ± 0.8	25 ± 4	7 ±	6 ± 3
cordovan (n = 3)	14 ± 2	162 ± 18	8 ± 2	8 ± 4	0 ± 2	4 ± 0.5
Hairbulbs (from 10-day-old mice)						
black (n = 5)	46 ± 21	538 ± 270	93 ± 72	131 ± 83	21 ± 31	10 ± 6
lethal yellow (n = 5)	12 ± 4	150 ± 57	0.3 ± 0.6	23 ± 7	0 ± 0.7	3 ± 1

The melanogenic activities of extracts of whole skin or hairbulbs were measured as described in Materials and methods,and are reported as means ± SEM in units of pmol/μg protein/h; backgrounds have been subtracted and were established using extraction buffer only.

have also shown that DHICA oxidase activity in extracts of *brown* melanocytes (melan-b) is markedly impaired compared to melanocytes wild-type at that locus (melan-a) and is lacking altogether in extracts of skin of *cordovan* mice. However, TRP1 immunopurified from *brown* mutant melanocytes (S91 melanoma) has virtually the same specific activity as wild type TRP1 (purified from B16 melanoma), although it should be noted that the protein concentrations of these immunopurified samples are so low as to make calculations of specific activities rather imprecise. Nevertheless, TRP1 function in the production of black rather than brown melanin could be regulated at other levels as well. Recently, Orlow *et al.* (1993) reported that in S91 (*brown*) melanoma cells, TRP1 was not fully glycosylated and was not appropriately delivered to melanosomes. This would suggest that even if brown mutation TRP1 has normal DHICA oxidase activity it may not be present in the melanosome wherein melanin production occurs. In other studies (Hearing *et al.*, 1992; Tsukamoto *et al.*, 1992b; Kobayashi *et al.*, 1994) we have shown that TRP1 plays an important structural role in the stabilization of melanogenic activities, and this function of TRP1 is also likely to be important to the quality and quantity of melanin produced in the wild-type and mutant cells. The fact that the point mutation in *brown* occurs in the motif (termed the EGF region) putatively responsible for protein:protein interactions (Jackson *et al.*, 1992), rather than in the catalytic domain, further supports the importance of such interactions to the production of melanins. The decreased tyrosinase activity evident in brown mutant cells (~40–50%, Tsukamoto *et al.*, 1992b; 1992c) is evidence of this interaction and the resulting decrease in overall melanin production undoubtedly also plays a role in resultant visible color. It has also recently been shown that one form of tyrosinase-positive albinism, termed OCA3, maps to the human analogue of the *brown* locus, and results from a mutation that eliminates the expression of TRP1 (Boissy *et al.*, 1993). The fact that a point mutation in TRP1 can have such a dramatic effect on tyrosinase activity and result in virtually no melanin production *in vivo* underscores the importance of the interactions of TRP1 with tyrosinase as an important melanogenic determinant.

Since TRP1 is involved in the oxidation and further metabolism of DHICA that is required for melanin polymerization, delivery of TRP1 to the melanosome may be an important regulatory control over melanogenesis as noted above. The presence of relatively high levels of DHICA in coated vesicles *en route* to the melanosome has been reported (Hatta *et al.*, 1988; Mishima, 1992; Shibata *et al.*, 1993) which demonstrates that tyrosinase is fully active in those organelles despite the fact that no melanin is produced there. There is also evidence (Orlow *et al.*, 1993) that the sorting of TRP1 occurs in coated vesicles independently from tyrosinase and TRP2, which in turn suggests that TRP1 may not be co-delivered to the melanosomes with those other melanogenic enzymes. This suggestion has been further supported by a recent study (Vijayasaradhi and Xu, 1993) which demonstrated that only the membrane binding region of TRP1 is necessary for its correct processing and delivery to the melanosome; this targeting sequence in TRP1 is significantly different from the analogous sequence in tyrosinase and TRP2. This being the case, it would provide for a natural delay in melanin production until all those enzymes are delivered to the melanin granule and become associated within the melanosomal complex (Hearing *et al.*, 1992; Kobayashi *et al.*, 1994; Orlow *et al.*, 1994). Following such association within the melanosome, DHICA generated in the coated vesicles could then be metabolized by TRP1 and melanin polymerization could ensue, followed shortly thereafter by visible pigmentation; such a time course and distribution of melanogenic activities has recently been reported by Wilczek and Mishima (1993). When TRP2 (DOPAchrome tautomerase) is deglycosylated, its enzymatic activity is significantly increased although its stability is decreased (Aroca *et al.*, 1992). Whether comparable effects can be demonstrated on the catalytic function of tyrosinase and/or TRP1 activity remains unknown, but this would obviously provide yet another level at which the quantity and quality of melanins produced can be regulated *in vivo*. In our other related study (Jiménez-Cervantes *et al.*, 1994), we have demonstrated a 3–5 h lag in the DHICA oxidase reaction catalyzed by LEMT, and there is little accumulation of melanin until a few h later. The role played by this inherent delay under *in vivo* conditions is an interesting matter for conjecture, especially with respect to the existence of significant quantities of DHICA in coated vesicles in transit to the melanosome. Future work will naturally be directed towards further characterizing this novel regulatory point in the melanogenic pathway and its importance to the structure and function of the melanins produced.

Materials and methods

Antibodies, cell lines and culture techniques

The generation and specificities of the rabbit polyclonal antisera (αPEP1 and αPEP7) against synthetic peptides corresponding to the carboxyl termini of TRP1 and tyrosinase, respectively, have been previously described (Jiménez *et al.*, 1989; 1991; Tsukamoto *et al.*, 1992b). The immortalized melanocyte cell lines melan-a (black, i.e. wild-type tyrosinase and wild-type TRP1), melan-b (brown, i.e. wild-type tyrosinase and mutant TRP1) and melan-c (albino, i.e. mutant tyrosinase and wild-type TRP1) used in this study were gifts of Dr Dorothy Bennett (St. George's Hospital Medical School; Bennett *et al.*, 1987, 1989). The B16 F10 and S91 murine melanoma cell lines used have also been previously described (Hearing *et al.*, 1985): B16 F10 melanoma cell line is a subline of the pigmented B16 melanoma (C57BL/6N, i.e. wild-type tyrosinase and wild-type TRP1) whereas the S91 murine melanoma was derived from DBA (brown) mice and therefore carries the germline homozygous mutation of the TRP1 gene (i.e. wild-type tyrosinase and mutant TRP1). 3T3 Swiss mouse fibroblasts and the derivation of transfected clones expressing tyrosinase or TRP1 have also been previously described (Winder, 1991; Winder *et al.*, 1993a,b). Cells were routinely grown to semiconfluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and other supplements (Jiménez *et al.*, 1988, 1991; Winder *et al.*, 1993b); they were harvested with brief treatment with trypsin−EDTA and used for subculturing or were further processed as detailed below.

Melanogenic assays

Tyrosine hydroxylase and DOPA oxidase activities were assayed as previously described (Tsukamoto *et al.*, 1992b; Kobayashi *et al.*, 1994). Assays to measure DHI or DHICA oxidase were always carried out at pH 6.8, 37°C for 60 min, and activities were measured by HPLC as the disappearance of those substrates from reaction mixtures compared with controls for spontaneous autooxidation (Tsukamoto *et al.*, 1992b; Aroca *et al.*, 1993). Data were converted to pmol by comparison with known standards of those intermediates and backgrounds were determined using either buffer or bovine serum albumin (BSA) in place of enzyme samples. The HPLC assay used was that described by Palumbo *et al.* (1987), and employs a hydrophobic C18 column and isocratic elution with 0.15 M sodium borate buffer, pH 2.5, containing 25% methanol at a flow rate of 0.5 ml/min. Elution of peaks was monitored by A280. Under these conditions, DOPA elutes at 5.2 min, tyrosine at 6.2 min, DOPAchrome at 8.5 min, DHI at 10.4 min and DHICA at 12.7 min. DHI and DHICA were kindly provided by Dr Giuseppe Prota (University of Naples, Naples) and were purchased commercially from Regis Chemical Co. (Morton Grove, IL).

DHICA oxidase was also measured by the MBTH assay, which was originally described by Winder and Harris (1991) for DOPA oxidase. Briefly, 100 μl sample was incubated at 37°C with 800 μl of MBTH-buffer solution (3.75 mM MBTH, 2.5% *N,N*-dimethylformamide, 125 mM potassium phosphate, pH 7.0) and 100 μl DHICA (0.5 mM DHICA, 1 mM EDTA). Monitoring of the absorbance (A_{470}) at 15 min intervals demonstrated a linear increase in absorbance from 15 to 75 min; rates of DHICA oxidation were corrected for background autooxidation and were calculated using least squares linear regression; ε = 15 000 mol/cm.

Metabolic labeling and immunoprecipitation, Western blotting

These techniques were performed as previously described (Jiménez *et al.*, 1989, 1991; Tsukamoto *et al.*, 1992b). Briefly, for metabolic labeling and radioimmunoprecipitation experiments, semiconfluent flasks of cells were labeled for 4 h with [^{35}S]methionine (0.2 mCi/ml, New England Nuclear). The cells were then harvested with trypsin and solubilized at 4°C for 60 min in 1% Nonidet-P40, 0.01% SDS, 0.1 M Tris−HCl, pH 7.2, 1 μg/ml aprotinin and 100 μM phenylmethylsulfonyl-fluoride. 1×10^8 d.p.m. of the ^{35}S-labeled extracts were incubated with 10 μl antibodies, complexed with 50 μl protein G−Sepharose (Pharmacia LKB, Piscataway, NJ), washed thoroughly, then the specifically bound immune complexes were separated by SDS gel electrophoresis (Laemmli, 1970), and visualized by autoradiography. For Western blotting, unlabeled extracts of cells or immunopurified proteins were separated by electrophoresis as above, transferred to nitrocellulose membranes, and reacted with antibodies. Visualization of antibody localization was performed using Enhanced ChemiLuminescence (Amersham Corp., Arlington Heights, IL).

Immunoaffinity purification of proteins

Immunoaffinity columns were prepared by covalently linking purified anti-peptide IgG (~10 mg) to protein A−Sepharose (5 ml) columns using IgG Orientation Kits (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions, and as detailed in Jiménez *et al.* (1991). B16 F10 or S91 melanomas growing subcutaneously in mice were removed by excision, homogenized and lysed in lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). The cells were then solubilized in 1% Nonidet P40, 0.1% SDS, 0.1 M Tris−HCl, pH 7.2, phenylmethyl-sulfonylfluoride and aprotinin overnight at 4°C. The samples were centrifuged at 10 000 *g* for 30 min at 4°C and identical aliquots (~50 mg protein) of the soluble supernatant fraction were bound to each immunoaffinity column; nonabsorbed proteins were washed through with 30 ml of binding buffer. Specifically absorbed proteins were then removed from the column with 10 ml of the Elution buffer supplied with the columns, and dialyzed versus 0.5% Nonidet-P40, 0.1 M Tris buffer, pH 7.2, 0.1 mM EDTA, with phenylmethyl-sulfonylfluoride and aprotinin, overnight at 4°C. They were subsequently concentrated using Centriprep 30 (Amicon Corp., Beverly, MA): typical yields of tyrosinase and TRP1 from 50 mg starting material was ~10 μg.

Transfection techniques

The transfection techniques used to generate the various 3T3 Swiss cell lines studied have been previously described (Winder, 1991; Winder *et al.*, 1993a,b). Briefly, 3T3 Swiss murine fibroblasts were grown in medium as detailed above, then co-transfected using the calcium phosphate method and 2 μg selectable marker (pKG4 or pBabehygro) and 18 μg of expression vector of choice (tyrosinase and/or TRP1). In doubly transfected cells, the stably transfected tyrosinase subline (termed clone *c*) was subsequently transfected with the TRP1 expression vector (pHDMT4) and pBabehygro (hygromycin resistance gene). Following subsequent selection of stably transfected cells with the appropriate antibiotic (G418 at 800 μg/ml and hygromycin at 175 μg/ml were used for selection, respectively), cloned cells were picked and grown for further study.

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