Tyrosinase Stabilization by Tyrp1 (the brown Locus Protein)*

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Mammalian melanogenesis is regulated directly or indirectly by over 85 distinct loci. The TyrlAlbino locus, in which mutations cause a lack of pigmentation, encodes tyrosinase (Tyr), the critical and rate-limiting melanogenic enzyme. Other melanogenic enzymes include Tyrp1 (or TRP1) and 3,4-dihydroxyphenylalaninechrome tautomerase (Dct or TRP2) encoded at the Tyrp1brown and Dct/slaty loci, respectively. Murine Tyrp1 can oxidize 5,6-dihydroxyindole-2-carboxylic acid (DHI-2CA) produced by Dct, but mutations in Tyrp1 also affect the catalytic functions of Tyr. All three enzymes are membrane-bound melanosomal proteins with similar structural features and are thought to interact within and stabilize a melanogenic complex. We have now further investigated the effect of a Tyrp1b mutation on Tyr stability. Pulse/chase labeling experiments show that Tyr is degraded more quickly in Tyrp1b mutant melanocytes than in melanocytes wild type at that locus. This reduced stability of Tyr can be partly rescued by infection with the wild type Tyrp1 gene, and this is accompanied by phenotypic rescue of infected melanocytes. In sum, these results suggest that, in addition to its catalytic function in oxidizing DHICA, Tyrp1 may play an important role in stabilizing Tyr, a second potential role in the regulation of melanin formation.

Tyrosinase-related protein 1 (Tyrp1, also known as TRP1 and gp75) is encoded by the Tyrp1brown locus, one of more than 85 genes that directly or indirectly affect coat color in mice (1–4). Tyrp1 is expressed specifically in melanocytes and functions in melanin synthesis within melanosomes, as do the other members of the tyrosinase-related protein (TRP) family, which includes Tyr (also known as tyrosinase), and Dct (also known as DOPAchrome tautomerase and TRP2). Although TRPs have many similar structural features, including a transmembrane region, two metal-binding regions, and a cysteine-rich epidermal growth factor motif thought to be involved in protein-protein interactions (8). Our and co-workers (20) have shown that Tyr, Tyrp1, and Dct co-purify from murine melanoma cells in high molecular weight aggregates, and our group has demonstrated that Tyr activity in vitro is more stable in the presence of Tyrp1 and/or Dct (21, 22). Thus we suggested that TRPs might interact in vivo and be stabilized in a multi-enzyme complex, which plays an important role in the regulation of mammalian melanogenesis. Because Tyrp1 is the most abundant glycoprotein expressed in melanocytes (23, 24), it may play a key role in forming that multi-enzyme complex.

There are various murine mutations at the Tyrp1 locus (e.g., Tyrp1b/light or Tyrp1c/cordovan) with known molecular lesions. (25, 26). Well known among them is the Tyrp1b/melanoma mutation, which results in the substitution of tyrosine for cysteine at position 86 (C86Y) within the epidermal growth factor domain thought to function in protein-protein interactions. This mutation causes proteins in vivo and in vitro to be stabilized in a multi-enzyme complex, which plays an important role in the regulation of mammalian melanogenesis. Because Tyrp1 is the most abundant glycoprotein expressed in melanocytes, we have investigated the effect of the Tyrp1 mutation on the stability of Tyr in vivo. We used the murine melanocyte line melan-b, which is homozygous for the Tyrp1b mutation, and the melan-a melanocyte line, wild type at this locus (28, 29). In this study, we compared the stability of Tyr in melan-b cells and in melan-a cells. Pulse labeling and chase experiments showed that Tyr is degraded more quickly in Tyrp1 mutant melan-b cells than in the wild type melan-a cells. Moreover, the lesser stability of Tyr in melan-b cells can be partly rescued by infection with the wild type Tyrp1 gene, along with phenotypic rescue of those cells. These results demonstrate that in addition to its catalytic function in DHICA oxidation, Tyrp1 also plays an important role in the stabilization of Tyr in melanocytes and might also indirectly contribute to the regulation of melanin production.

The abbreviations used are: TRP, tyrosinase-related protein; DHI, 5,6-dihydroxyindole; DHICA, DHI-2-carboxylic acid; DOPA, 3,4-dihydroxyphenylalanine; Tyr, tyrosinase; Dct, DOPAchrome tautomerase.
**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—Melanocyte lines cultured from black (Tyrr1/Tyrr1) and from brown (Tyrr1b/Tyrr1b) nonagouti mice (melan-a and melan-b cells, respectively) were cultured as previously reported (28, 29). NIH3T3 murine fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. 

**Antibodies Used**—The peptide antibodies used in this study, aPEP1, aPEP7, aPEP8, and aPEP13, were generated in rabbits against synthetic peptides corresponding to the carboxyl termini of murine melanogenic proteins Tyrr1, Tyr, Dct, and Pmel17 (also known as the silver protein), respectively, and specifically recognize each melanogenic protein as described previously (9, 12, 30). Another antibody used in this study, aP5, was produced against a chro-mo-terminal peptide of murine Tyr, an epitope recognized only following denaturation, as detailed previously (12).

**Metabolic Labeling and Immunoprecipitation**—These techniques were performed as reported previously (9, 12, 13, 17, 30, 31). Briefly, semiconfluent cells were preincubated in methionine-free medium containing dialyzed fetal bovine serum, pulsed at 30 min with [35S]methionine (0.1–1.0 μCi/ml) (NEN Life Science Products) in methionine-free medium, and chased in complete medium for 0–48 h, as detailed in the figure legends. The cells were washed with phosphate-buffered saline without Mg2+ and Ca2+ and solubilized at 4 °C for 60 min in Nonidet P-40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, 100 μM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin). The extracts were centrifuged for 15 min at 10,000 × g, and the supernatants were kept at 4 °C for immunoprecipitation. The resulting [35S]-labeled extracts were then preclreared with 10 μl of normal rabbit serum and 100 μl of protein G-Sepharose 4FF (Amersham Pharmacia Biotech). Two × 106 trichloracetic acid-precipitable cpm of preclreared extracts were incubated with 5 μl of antibodies for 1 h at 4 °C and were then complexed with 30 μl of protein G-Sepharose for 30 min at 4 °C. The immune complexes were washed six times with Nonidet P-40/SDS buffer, eluted in SDS sample buffer containing 2-mercaptoethanol at 95 °C for 3 min and analyzed by SDS gel electrophoresis (32), followed by fluorography. For immunoprecipitation using aPEP5 antibody, labeled cell extracts were denatured before incubation with antibody as reported previously (12). For quantification of immunoprecipitated bands, fluorographs were analyzed with BioImage (Milibore). 

**Infection of the Wild Type Tyrr1 Gene into Melan-b Melanocytes**—Retroviral infection of melan-b cells was performed as described previously (33). Briefly, psi-2 helper fibroblasts transfected with the Tyrr1 retrovirus (pHS-TRP1) and with pSV2-neo were selected in G418 (200 μg/ml) and were incubated for 12 h in melanocyte medium without 12-O-tetradecanoylphosphol 13-acetate and 10 μM 12-O-tetradecanoylphosphol 13-acetate. The conditioned medium containing virus was harvested, filtered through a 22-μm membrane, supplemented with 200 nM 12-O-tetradecanoylphosphol 13-acetate and 10 μg/ml polybrene, and placed in melan-b cultures plated the previous day at 2 × 104 cells/ml. The medium was replaced after 12 h with freshly prepared conditioned medium. After three such exposures to the conditioned medium, cells were cultured in fresh melanocyte medium for 2 days and were then subcultured and subcloned.

**Southern Blotting Analysis**—Southern blotting was performed with some modification as described previously (33). Briefly, DNA was isolated from melanocytes with a Qiagen DNA isolation kit, according to the manufacturer’s instructions. SacI-digested DNA was separated by agarose gel electrophoresis, transferred to a Hybond-N membrane (Amersham Pharmacia Biotech) and hybridized with a fluorescein-labeled HindIII-digested pM70 probe for the murine Tyrr1 gene (10, 34), followed by visualization with a Fluorescein Gene Image detection system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

**RESULTS**

**Quantification of Pulse-labeled Tyr by Immunoprecipitation with aPEP7**—To quantify the degradation of Tyr within melanocytes by pulse/chase metabolic labeling, Tyrr1 melanocytes metabolically labeled melan-a melanocyte extract was specifically immunoprecipitated with aPEP7 antibody, separated by SDS gel electrophoresis, visualized by fluorography, and densitometrically analyzed (Fig. 1). An excellent correlation was obtained between the relative intensity of the immunoprecipitated Tyr and the amount of melanocyte cell extract added, showing that the degradation of Tyr can be quantified using such immunoprecipitation analysis.

**Degradation of Tyr in Melan-a and Melan-b Melanocytes**—The synthesis and degradation of melanogenic proteins in melan-b (Tyrr1b/Tyrr1b) and melan-a (Tyrr1/Tyrr1) melanocytes was compared using peptide antibodies specific for each protein (Fig. 2A). As found previously (9, 12, 17), two Tyr bands from melan-a melanocytes were observed, a higher broad band and a lower band, representing glycosylated and de novo Tyr, respectively. In melan-a and in melan-b melanocytes, Dct and Pmel17 (also known as gp100 and the silver protein) were expressed and degraded similarly. In contrast, Tyrr1 in mutant melan-b cells was not glycosylated to a significant degree and disappeared more quickly than in wild type melan-a melanocytes. Tyr was also underglycosylated to some extent and was degraded significantly more quickly in melan-b cells than in melan-a cells, even though both types of cells produce genetically identical Tyr. Densitometric analysis of three independent pulse/chase experiments showed clearly that Tyr and Tyrr1 in melan-b cells were degraded significantly more quickly than in melan-a cells, whereas rates of degradation of Dct and Pmel17 were virtually identical (Fig. 2B). As the rate of processing of Tyr from the de novo type (i.e. the lower molecular weight band) to the highly glycosylated type (i.e. the higher molecular weight band) was decreased in melan-b cells compared with melan-a cells, we analyzed the degradation of the glycosylated Tyr band in melan-a and melan-b cells (Fig. 2C). The amount of glycosylated Tyr present at the 1.5-h chase period was adjusted to 100% as a control, and it is clear that even glycosylated Tyr was degraded significantly more quickly in melan-b cells than in melan-a cells.

Tyr is inserted and anchored in the melanosomal membrane via its transmembrane region (near the carboxyl-terminal) and
can be readily digested proteolytically there to produce a smaller Tyr with a truncated carboxyl terminus but which still retains enzymatic activity (12, 35). Therefore, it was possible that only the carboxyl terminus of Tyr might have been lost during the pulse/chase experiments reported above, which might be misleading when using only the aPEP7 antibody (which recognizes that carboxyl terminus). Therefore, we also immunoprecipitated Tyr using aPEP5 (which recognizes the amino terminus of Tyr) and analyzed its degradation in melan-a and melan-b cells. Again, a decrease in glycosylation and a significantly lowered stability of Tyr in melan-b cells was observed (Fig. 3), indicating that the loss of immunoreactivity represents degradation of the entire protein and not just its carboxyl terminus.

Tyr Stability in Melan-b Melanocytes Infected with Wild Type Tyrp1—We next compared the degradation of Tyr in uninfected and in Tyrp1-infected melan-b melanocytes. When melan-b cells were infected with the wild type Tyrp1 gene and cultured for 10 days, some black cells (i.e. phenotypically rescued cells) were observed among the infected cell population, as reported previously (33). Such cells represented about 20% of the total infected melan-b cell population. We subcloned these cells by limited dilution cloning in 96-well plates and established several cloned lines. Clones 1 and 2 were produced from mock-infected melan-b cells, whereas clones 3, 4, and 5 were from Tyrp1-infected melan-b cells. The relatively long term culture of cloned Tyrp1-infected cell lines without selective pressure caused the emergence of a heterogeneous population, including some cells that appeared brown again. Southern blotting analysis (Fig. 4, inset) demonstrated that uninfected clones 1 and 2 did not contain wild type Tyrp1, as expected, whereas clones 3, 4, and 5 contained the infected wild type Tyrp1 minigene. This
is consistent with the phenotypes of their cell pellets, i.e. pellets of clones 1 and 2 were brown, whereas pellets of clones 3, 4, and 5 were black. When degradation of Tyr in these subcloned cells was analyzed with pulse/chase labeling, Tyr was significantly more stable following infection of melan-b melanocytes with the wild type Tyrp1 gene (Fig. 4).

**DISCUSSION**

This study demonstrates that Tyr (encoded at the Tyr/Albino locus) is degraded more quickly in melan-b melanocytes, which are homozygous for the brown mutation (Tyrp1<sup>b</sup>/Tyrp1<sup>b</sup>), than in melan-a melanocytes, wild type at that locus. Moreover, the reduced stability of Tyr and the brown phenotype of melan-b melanocytes could be rescued by infection with the wild type Tyrp1 gene. Thus, the decreased stability of Tyr in melan-b cells is caused by the C86Y point mutation in the Tyrp1 gene. Our previous study (22) showed clearly that Tyr catalytic function in melan-b cells is only ~65% that found in melan-a cells, whereas Dct function (as DOPAchrome tautomerase) is identical in both cell lines. Several clues about the important role of Tyrp1 in Tyr function have also been provided by in vivo studies. Tyr catalytic function in the skin of brown mice is only 80% that found in the skin of black mice (36), and melanin content of brown mouse hair is only 30–35% that found in black mouse hair (19, 36).

Because the only difference between these black and brown melanocytes (and animals) is the point mutation in the Tyrp1 gene, how can the instability of Tyr in Tyrp1-mutant melanocytes be explained? One attractive possibility is that the brown mutation affects the direct interaction between Tyr and Tyrp1 and suppresses the stabilizing influence of Tyrp1 on Tyr. Current studies by our group and others have demonstrated that Tyr and Tyrp1 are localized in a melanosomal enzyme complex and interact with each other (20, 21). The brown mutation (C86Y) results in a structural change in the epidermal growth factor motif of Tyrp1, a domain thought to play an important role in protein-protein interactions (25). The mutation appears to allow the rapid degradation of under-glycosylated Tyrp1, resulting in the absence of Tyrp1 in melanosomes (27), which would in turn reduce direct interaction of Tyrp1 with Tyr. Decreases in the glycosylation of Tyrp1 and Tyr suggest that these two proteins might interact in some manner in the Golgi apparatus where such glycosylation occurs. The altered glycosylation might affect proper routing of these proteins to the melanosome and might thus also be an important factor in their rapid degradation.

Mutations in Tyrp1 also lead to structural changes in mela-
nosomes (36–40), which might, at least in part, result from the reduced stability of Tyr and Tyrp1 observed in this study. Melanosomes produced in brown melanocytes (i.e. Tyrp1<sup>b</sup>/Tyrp1<sup>b</sup>) are round, particulate, and relatively disorganized in structure, rather than ovoid, lamellar, and regular structures as normally observed on a eumelanogenic background. Further, other mutations at the Tyrp1 locus (e.g. Tyrp1<sup>h1</sup>) cause the premature death of follicular melanocytes coordinated with melanogenesis, suggesting that Tyrp1 functions in some manner to protect cells from toxic intermediates during melanin biosynthesis (26). One possible mechanism for Tyr degradation within the melanosome might be its susceptibility to attack and cleavage by melanin intermediates (such as quinones) in melanosomes of Tyrp1 dysfunctional melanocytes.

Infection of the wild type Tyrp1 gene into melan-b melanocytes resulted in only a partial rescue of Tyr instability. Even in subcloned Tyrp1-infected cells, Tyrp1 was not fully glycosylated and disappeared relatively quickly, as it did in uninfected melan-b cells (data not shown). Incomplete rescue of Tyr stability in melan-b melanocytes by Tyrp1 gene infection might result from insufficient Tyrp1 expression and/or function to fully stabilize Tyr as it does in wild type melan-a cells. A further complication is that reversion to the parental brown phenotype occurs rather quickly, which may result from methylation of viral DNA (33). It seems likely that, were it possible to analyze the stability of Tyr solely in cells expressing infected wild type Tyrp1, rescue of Tyr stability would be significantly more dramatic.

It is not completely clear how the decreased stability of Tyr in Tyrp1 mutant melanocytes contributes to the brown phenotype. The decrease in Tyr activity necessarily results in a decrease in melanin production, as has been shown for human and mouse brown hair. Failure of routing of Tyrp1 to melanosomes in Tyrp1 mutant melanocytes must eliminate Tyrp1 function(s) in melanosomes, including its catalytic activity. In humans, one type of human TYR-positive albinism, termed OCA3, has been identified recently that maps at the human homolog of the Tyrp1/brown locus (41). Melanosomes isolated from that OCA3 patient have no Tyrp1 expression, and although the transcription and translation of TYR is detected at normal levels, melanin production is diminished drastically, similar to what is observed in melan-b melanocytes. Human TYRP1 does not seem to have DHICA oxidase activity (42), and thus the TYR stabilizing function of TYRP1 may in general be even more important to the regulation of mammalian pigmentation than is its catalytic function. Human TYR is significantly less stable than murine Tyr (7),<sup>2</sup> and the stabilizing role of Tyrp1 on Tyr may play a more active and essential role in the regulation of human pigmentation than in mouse pigmentation. Further studies on TYR stability in OCA3-derived and normal human melanocytes will be necessary to clarify this point. In sum, these results suggest that in addition to its catalytic function(s), the principal function of Tyrp1 may be to stabilize Tyr in melanosomes, and Tyrp1 might thus regulate basal levels of melanin formation.

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<sup>2</sup>T. Kobayashi, G. Imokawa, D. C. Bennett, and V. J. Hearing, unpublished results.

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