Direct interaction of tyrosinase with Tyrp1 to form heterodimeric complexes in vivo

Takeshi Kobayashi[1] and Vincent J. Hearing[2,*]

[1]Department of Physiology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan
[2]Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: hearingv@nih.gov)

Accepted 13 October 2007
Journal of Cell Science 120, 4261-4268 Published by The Company of Biologists 2007
doi:10.1242/jcs.017913

Summary

Mutations of the critical and rate-limiting melanogenic enzyme tyrosinase (Tyr) result in hypopigmentation of the hair, skin and eyes. Two other related enzymes, Tyrp1 and Dct, catalyze distinct post-Tyr reactions in melanin biosynthesis. Tyr, Tyrp1 and Dct have been proposed to interact with and stabilize each other in multi-enzyme complexes, and in vitro, Tyr activity is more stable in the presence of Tyrp1 and/or Dct. We recently reported that Tyr is degraded more quickly in mutant Tyrp1 mouse melanocytes than in wild-type Tyrp1 melanocytes, and that decreased stability of Tyr can be partly rescued by infection with wild-type *Tyrp1*. Although interactions between Tyr and Tyrp1 have been demonstrated in vitro, there is no direct evidence for Tyr interaction with Tyrp1 in vivo. In this study, we use in vivo chemical crosslinking to stabilize the association of Tyr with other cellular proteins. Western blot analysis revealed that Tyrp1, but not Dct, associates with Tyr in murine melanocytes in vivo, and more specifically, in melanosomes. Two-dimensional SDS-PAGE analysis detected heterodimeric species of Tyr and Tyrp1. Taken together, these data demonstrate that Tyrp1 interacts directly with Tyr in vivo, which may regulate the stability and trafficking of melanogenic enzymes and thus pigment synthesis.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/24/4261/DC1

Key words: Tyrosinase, Tyrp1, Heterodimer, Melanosome, Crosslinking

Introduction

In mammals, melanin is produced only in melanocytes, which generate intracellular organelles (termed melanosomes) that are specialized for melanin synthesis and deposition. Melanin biosynthesis is mediated by a group of enzymes uniquely expressed in melanocytes, called the tyrosinase-related protein (TRP) family. The TRP family has three known members: tyrosinase (Tyr), Tyrp1 (originally termed TRP1), and Dct (originally termed TRP2) (Bennett, 1991; Hearing and Tsukamoto, 1991; Kobayashi et al., 1995; Marks and Seabra, 2001). These three enzymes are type I membrane glycoproteins with significant structural homology, and all are targeted to and located in the melanosomal membrane. They act within the context of a series of reactions in the melanogenic pathway to control melanin production in melanosomes (Hearing, 1999; Hearing, 2005; Wang and Hebert, 2006). Tyr is encoded at the *albino* locus and is the critical and rate-limiting enzyme required for melanogenesis, catalyzing the initial reaction of tyrosine hydroxylation. Mutations in *Tyr* cause reduced or absent pigmentation of hair, skin and eyes, an inherited hypopigmentary disease in humans known as oculocutaneous albinism (OCA). Tyrp1 and Dct, encoded at the *brown* and *slaty* loci, respectively, have distinct catalytic functions in melanin synthesis downstream of Tyr. Murine Tyrp1 oxidizes a downstream melanogenic intermediate (5,6-dihydroxyindole-2-carboxylic acid, DHICA) as does Dct, which functions as DOPAchrome tautomerase (Shibahara et al., 1986; Jackson, 1988; Jackson et al., 1992; Tsukamoto et al., 1992a; Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994b). In contrast to mutations in Tyr, mutations in Tyrp1 or Dct affect the quality of melanin synthesized rather than the quantity. Melanin produced by brown (Tyrp1-mutant) mice is less polymerized compared with that in black mice (Ozeki et al., 1995), whereas slaty (Dct-mutant) mice have reduced levels of DHICA-containing melanin (Tsukamoto et al., 1992b; Ozeki et al., 1995). In humans, mutations in TYR result in the most severe form of OCA (OCA1) whereas mutations in TYRP1 cause a less severe form of the disease (OCA3) (Boissy et al., 1996; King et al., 2003). Mutations in genes encoding two other pigment-specific proteins, P and MATP, also affect tyrosinase function but do so by misdirecting the trafficking of tyrosinase to melanosomes, and give rise to OCA2 and OCA4, respectively (Costin et al., 2003).

Tyrp1 and Dct, in addition to having enzymatic functions, stabilize Tyr (Hearing et al., 1992; Winder et al., 1994; Kobayashi et al., 1994c), and coexpression of Tyr with Tyrp1 or Dct in melanocytes increases pigmentation (Zhao et al., 1996; Manga et al., 2000; Hirosaki et al., 2002). These results suggest that TRP family proteins associate and form multi-enzyme complexes on the internal surface of the melanosomal membrane. This hypothesis is supported by other studies. For example, large molecular complexes containing TRPs have been found in lysates of melanocytes (Orlow et al., 1994), whereas coprecipitation with crosslinkers has detected the association of TRPs (Toyofuku et al., 2001; Wu and Park, 2003). Furthermore, fluorescence resonance energy transfer and gel chromatographic analyses have demonstrated the association of Tyr and Tyrp1 (Jiménez-Cervantes et al., 1998). These results suggest that TRPs form complexes in vitro, but there is no direct evidence for intermolecular interactions of

TRPs in vivo because these studies examined TRPs only after solubilization of the cells.

Tyrp1 is one of most abundant glycoproteins in melanocytic cells (Tai et al., 1983; Vijayasaradhi and Houghton, 1991), and thus is likely to play an intrinsic role in the formation and stabilization of melanogenic enzyme complexes. We previously examined the stability of melanogenic proteins in mouse melan-b melanocytes [mutant at the *brown* (Tyrp1) locus] compared with melan-a melanocytes (wild type at that locus) (Kobayashi et al., 1998). That study revealed that Tyr degradation in melan-b cells is significantly quicker than in melan-a cells, even though both types of melanocytes have identical genetic backgrounds except at the *brown* locus. This reduced stability of Tyr could be partly rescued by wild-type Tyrp1, which suggested that Tyrp1 stabilizes Tyr in vivo. We have also shown that Tyrp1 functions as a molecular chaperone for Tyr in the endoplasmic reticulum (ER), which suggests that interactions of Tyrp1 with Tyr occur in the ER (Toyofuku et al., 2001). However, those earlier studies did not address whether Tyr interacts with Tyrp1 in post-ER compartments such as the Golgi and/or melanosomes in vivo. In our previous study (Kobayashi et al., 1998), Tyr was degraded more quickly in Tyrp1-mutant cells even with long chase times (6-48 hours) when melanogenic enzymes should have been delivered to melanosomes, which suggests that interactions of Tyr with Tyrp1 also occur in melanosomes. However, there is no direct evidence that Tyrp1 interacts with Tyr in melanocytes and/or in melanosomes in vivo where Tyr function is critical for melanin synthesis.

In this study, we used a chemical approach to crosslink intracellular molecular complexes in vivo. We now demonstrate that Tyr and Tyrp1 can be crosslinked and stabilized in melanocytes in vivo as well as in melanosomes. Two-dimensional PAGE analysis showed significant amounts of heterodimeric molecular complexes of Tyr and Tyrp1 in lysates of crosslinker-treated cells, revealing that Tyr associates directly with Tyrp1 in vivo.

Results

Guinea pig antibodies are specific for melanosomal proteins

To determine whether Tyrp1 interacts directly with Tyr in vivo, we stabilized intermolecular associations between proteins in melanocytes using a chemical crosslinker. The crosslinker used was DSP [dithiobis(succinimidylpropionate)], a homo-bifunctional *N*-hydroxy-succinimide ester with a ~12Å spacer arm, which is membrane permeable and can reach intracellular and intraorganellar spaces. Bonds crosslinked by DSP can be subsequently cleaved by thiols so that, after immunoisolation of crosslinked complexes, they can be dissociated and characterized. Thus, we solubilized cellular proteins from melanocytes pretreated with or without DSP, then immunoprecipitated Tyrp1 and/or Tyr from the cell extracts, and examined the linked proteins in immunoprecipitates using western blot analysis.

To assist in the characterization of those crosslinked complexes, we generated antisera to Tyr, Tyrp1, Dct and Silver (also known as Pmel17/gp100)in guinea pigs (Fig. 1A) to use along with our specific rabbit antisera to those proteins. By using guinea pig antibodies in western blots to analyze crosslinked molecular complexes immunopurified using rabbit antibodies, we could avoid crossreactivities of secondary antibodies and could thus specifically identify melanogenic proteins in complexes with little background due to IgG.

Western blot analysis of immunopurified or immunodepleted proteins from extracts of melan-a melanocytes revealed that the guinea pig antibodies specifically recognized their antigenic proteins as expected (Fig. 1A, left and middle). Melan-a melanocytes were wild type at the *Tyr*, *Tyrp1* and *Dct* loci, melan-b melanocytes were mutant at *Tyrp1* but were wild-type at the other two loci, whereas melan-c melanocytes were mutant at *Tyr*, but were wild-type at the other two loci. Altered mobilities of Tyr in melan-c cells and of Tyrp1 in melan-b cells reflected the altered processing of these mutant proteins (Fig. 1A, right), but all other proteins in the three extracts had comparable reactivity patterns. Confocal immunohistochemical analysis of Tyr and Tyrp1 localization in melan-a melanocytes using rabbit and guinea pig antisera showed that Tyr colocalized significantly with Tyrp1 in intracellular vesicles (Fig. 1B).

Interaction of Tyr and Tyrp1 in melanocytes

Since our previous study (Kobayashi et al., 1998) suggested that Tyrp1 associates with and stabilizes Tyr in vitro, we then examined whether Tyr interacts with Tyrp1 in melanocytes in vivo. We used rabbit antisera to immunopurify Tyr or Tyrp1 from extracts of melan-a melanocytes that had been pretreated with or without DSP, and then analyzed the resulting immune complexes by western blot using guinea pig antibodies (Fig. 2). When immunopurified with the Tyr antibody from cells untreated with DSP, only Tyr was detected (Fig. 2, lane 5). However, when DSP was used to crosslink proteins, significant levels of Tyrp1 coprecipitated with Tyr (Fig. 2, lanes 6, 7). The amount of Tyrp1 coprecipitated increased in a DSP dose-dependent manner. Similarly, when Tyrp1 was immunopurified with the Tyrp1 antibody, Tyr was detectable when cells had been treated with DSP but not in the absence of DSP (Fig. 2, lanes 8-10). Since coprecipitation of Tyr and Tyrp1 was not detectable in the absence of DSP, we conclude that they do not form complexes during solubilization or immunoprecipitation. The DSP-dependent coprecipitation of Tyr and Tyrp1 clearly indicates that they interact in melanocytes in vivo. Although western blotting is not strictly quantitative, we titrated the reactivities of these peptide antibodies to known amounts of cell extracts and then extrapolated those band intensities (see below and supplementary material Fig. S1). The results showed that Tyrp1 is about ten times as abundant in murine melanocytes as is Tyr (as expected), and that <10% of total Tyr is crosslinked with Tyrp1 in the complexes under these conditions. In related experiments, we used other chemical crosslinkers, including DSS, BS3 and DTSSP, but the most efficient crosslinking of Tyr-Tyrp1 was obtained with DSP (data not shown).

Tyr and Tyrp1 complexes are found in melanosomes

Next we examined whether Tyrp1 interacts with Tyr within melanosomes. We purified melanosomes from melanocytes (Seiji et al., 1963; Kobayashi et al., 1994a; Potterf et al., 1996) and performed similar crosslinking experiments with DSP (Fig. 3). Western blot analysis of immunoprecipitated and coprecipitated proteins from extracts of purified melanosomes revealed that when melanosomes were pretreated with DSP,

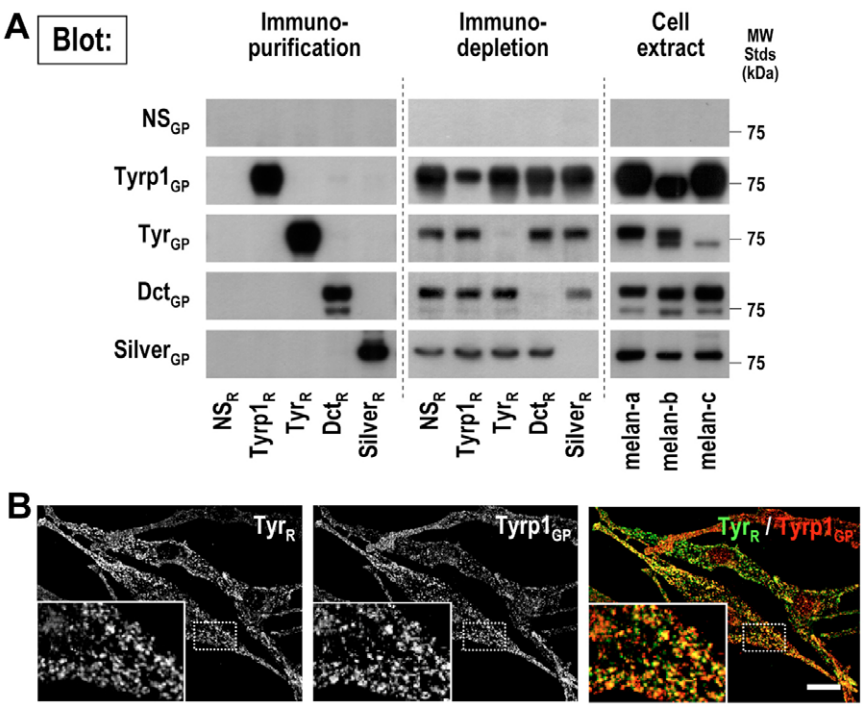

Fig. 1. Specificity of guinea pig antibodies for melanosomal proteins. (A) Analysis of antibody reactivities by immunopurification, immunodepletion and western blot analysis of melanocyte extracts. Tyr, Tyrp1, Dct or Silver proteins were immunopurified (left) from an extract of melan-a melanocytes using antibodies as noted at the bottom and then were analyzed by western blot using antibodies as noted on the left. Subscripts of 'GP' or 'R' refer to antisera generated in guinea pigs or rabbits, respectively; NS, normal serum. An extract of melan-a melanocytes was immunodepleted with antibody and protein-G-Sepharose (middle). Reactivity could be blocked by pre-incubation with the corresponding antigenic peptide (data not shown). Protein extracts from melan-a, melan-b or melan-c melanocytes were separated by SDS-PAGE (right). (B) Intracellular distribution of Tyr and Tyrp1 detected immunohistochemically. Melan-a melanocytes were fixed in 4% paraformaldehyde and stained for Tyr and Tyrp1. Tyrp1-positive (red) vesicles that colocalized with Tyr (green) are seen as yellow in the merged image. (Note that a relatively small fraction of vesicles is yellow, which may reflect cell variability in gene expression or antigen masking in melanized melanosomes.) Bar, 10 μm.

Tyrp1 and Tyr complexes could be detected. By contrast, coprecipitation of Tyr and Tyrp1 was not observed in the absence of DSP. These results show that Tyrp1 interacts with Tyr in melanosomes.

Tyr and Tyrp1 form complexes in cycloheximide-treated melanocytes

Since cycloheximide inhibits de novo protein synthesis, Tyr or Tyrp1 would be depleted in the ER and Golgi after cycloheximide treatment for appropriate periods. Previous studies have shown that post-translational processing of Tyr and Tyrp1 is essentially complete within 2 hours (Jiménez et al., 1988; Kobayashi et al., 1998), suggesting that they should be processed out of the ER and Golgi in that time frame. We used treatment with cycloheximide to examine whether Tyr interacts with Tyrp1 in organelles besides the ER and Golgi. We used YFP-markers to identify the ER and Golgi in melanocytes, and confirmed that although Tyr and Tyrp1 were abundant in the ER and Golgi of untreated melanocytes, they were not detectable in the ER or Golgi of melanocytes treated with 10 μg/ml cycloheximide for 10 hours (Fig. 4A). However, Tyr and Tyrp1 colocalized in most pigmented stage III and IV melanosomes (as seen in the bright field images) after treatment with cycloheximide. When melanocytes treated with or without cycloheximide for 10 hours were solubilized, immunopurified and examined by western blot (Fig. 4B), coprecipitation of Tyr and Tyrp1 was seen only in DSP-crosslinked cells, although the level of coprecipitated proteins was decreased slightly by cycloheximide (as would be expected because of the decreased protein synthesis). Since EndoH sensitivity is a specific criterion for whether proteins have been processed through the ER to the Golgi, we digested the DSP-linked complexes with EndoH and then analyzed them by western blot (data not shown). The results showed that the Tyr and Tyrp1 in those complexes had EndoH-sensitive and EndoH-resistant forms.

These experiments with DSP revealed that interactions between Tyr and Tyrp1 occur in the ER, Golgi and melanosomes, but it remained unclear whether Tyrp1 associates with Tyr directly or indirectly, although a preference for heterodimers of Tyr and Tyrp1 in vitro has been reported (Jiménez-Cervantes et al., 1998). To clarify this point, two-dimensional SDS-PAGE analysis was used to characterize the nature of the DSP-stabilized molecular complex of Tyr and Tyrp1. Crosslinked molecular complexes were immunoprecipitated by anti-Tyr from an extract of melan-a melanocytes pretreated with DSP, and were then separated by SDS-PAGE under non-reducing conditions in the first dimension (Fig. 5, from right to left). The proteins were then separated in the second dimension by SDS-PAGE under

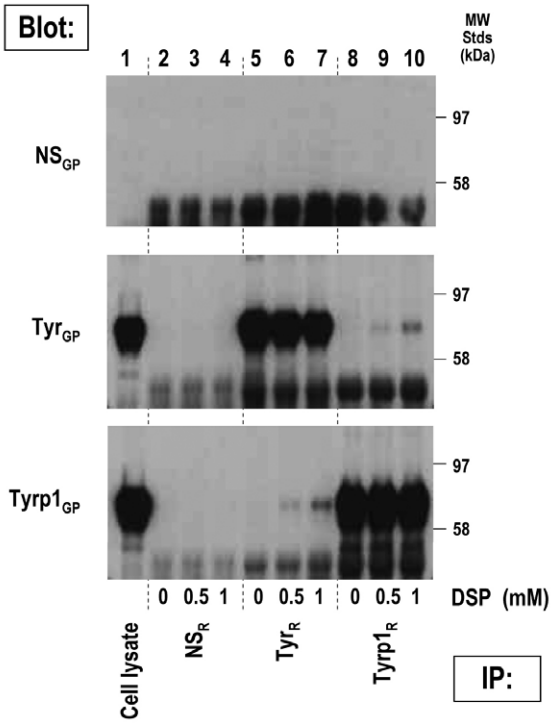

Fig. 2. Interaction of Tyr and Tyrp1 in melanocytes. Melan-a melanocytes were incubated in ice-cold PBS containing 0, 0.5 or 1 mM DSP (as indicated) for 15 minutes on ice, followed by quenching with 2 mM glycine/PBS and exhaustive washing with PBS. After solubilization, equal amounts of each cell extract were immunoprecipitated and melanogenic proteins were analyzed by SDS-PAGE and immunoblotting. Immunoprecipitated and coprecipitated proteins were separated by SDS-PAGE under reducing conditions, which cleaves the crosslinks, and proteins migrate at their original molecular sizes. Subscripts of 'GP' or 'R' refer to antisera generated in guinea pigs or rabbits, respectively; NS, normal serum.

reducing conditions, which cleaved the conjugates and revealed which molecules had composed the crosslinked complexes (Fig. 5, from top to bottom). When the blots were stained for Tyrp1, coprecipitated Tyrp1 was detected in extracts of cells pretreated with DSP, which migrated at ~150 kDa in the first dimension (Fig. 5, bottom right panel). Tyr was detected as a relatively minor spot at the same position (marked by the arrowhead) along with two major spots that were also observed in the untreated control (Fig. 5, middle panels). These results indicate that Tyrp1 interacts directly with Tyr in cells. Since coprecipitated Tyrp1 was not detected anywhere else, most of the coprecipitated Tyrp1 molecules had been released from crosslinked ~150 kDa heterodimers of Tyr and Tyrp1. Therefore, it is likely that Tyrp1 interacts with Tyr directly, even in melanosomes, although the two-dimensional SDS-PAGE analysis was performed only using whole cells.

Dct and Silver do not form complexes with Tyr or Tyrp1

We also examined whether Tyr and/or Tyrp1 were associated with the other known melanosomal proteins, Dct and Silver. When the blots were stained using anti-Dct or anti-Silver antibodies to identify coprecipitated proteins, neither of these proteins could be detected as a coprecipitate with Tyr or Tyrp1, even in melanocytes treated with DSP (Fig. 6). These results

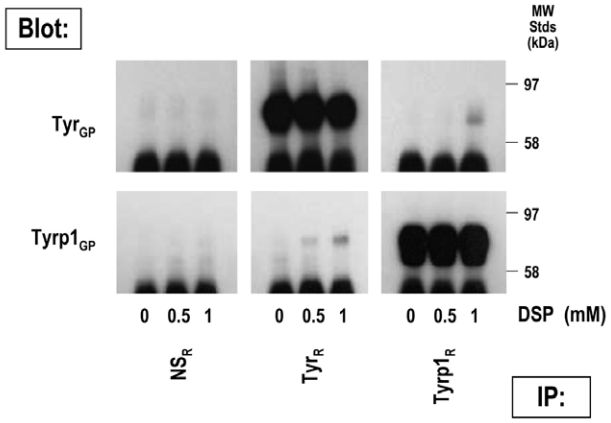

Fig. 3. Interaction of Tyr and Tyrp1 in melanosomes. Melanosomes were purified from melan-a melanocytes. Interaction of Tyr and Tyrp1 was analyzed by chemical crosslinking, immunoprecipitation and immunoblotting, as described for Fig. 2.

indicate that Dct and Silver do not form complexes with Tyr or Tyrp1 in vivo.

Interaction of Tyr with Tyrp1 is disrupted in *Tyrp1b*-mutant melanocytes

We had previously shown that Tyr was degraded more rapidly in *Tyrp1*^{b} mutant (melan-b) melanocytes compared with black (melan-a) melanocytes, which suggests that mutant Tyrp1 does not interact with Tyr (Kobayashi et al., 1998). As shown in Fig. 7, Tyr did not coprecipitate with mutant Tyrp1 in melan-b melanocytes, even when they had been pretreated with DSP. Conversely, when Tyr was immunoprecipitated from DSP-treated melan-b melanocytes, a faint band of Tyrp1 could be coprecipitated. However, that band was detectable even in the absence of DSP, which indicates that it was not DSP dependent. Therefore, we conclude that mutant Tyrp1 does not interact with Tyr in *Tyrp1*^{b} mutant melanocytes in vivo as it does in melanocytes that are wild type at that locus.

Discussion

In this study, we show that treatment of melanocytes in vivo or of purified melanosomes with the chemical crosslinker, DSP, induces the formation of stable complexes of Tyr and Tyrp1. Judging from the length of the spacer arm of DSP (~12Å), the formation of crosslinked Tyr-Tyrp1 complexes indicates that these molecules are very close together and that they associate in complexes in melanocytes and in melanosomes. Moreover, two-dimensional SDS-PAGE analysis demonstrated that the crosslinked complexes containing Tyr and Tyrp1 are heterodimers, indicating that Tyrp1 interacts directly with Tyr. Furthermore, these complexes can be detected in the ER, the Golgi and in early and late melanosomes.

Several groups have previously reported that Tyr and Tyrp1 associate with each other in vitro and form high molecular mass complexes (Orlow et al., 1994; Jiménez-Cervantes et al., 1998; Toyofuku et al., 2001; Wu and Park, 2003). However, those studies reported only on protein complexes after extraction from cells detected by various techniques, such as coimmunoprecipitation, cosedimentation, gel chromatography or fluorescence resonance energy transfer. Our study is the first to provide direct evidence that Tyrp1 interacts with Tyr in vivo.

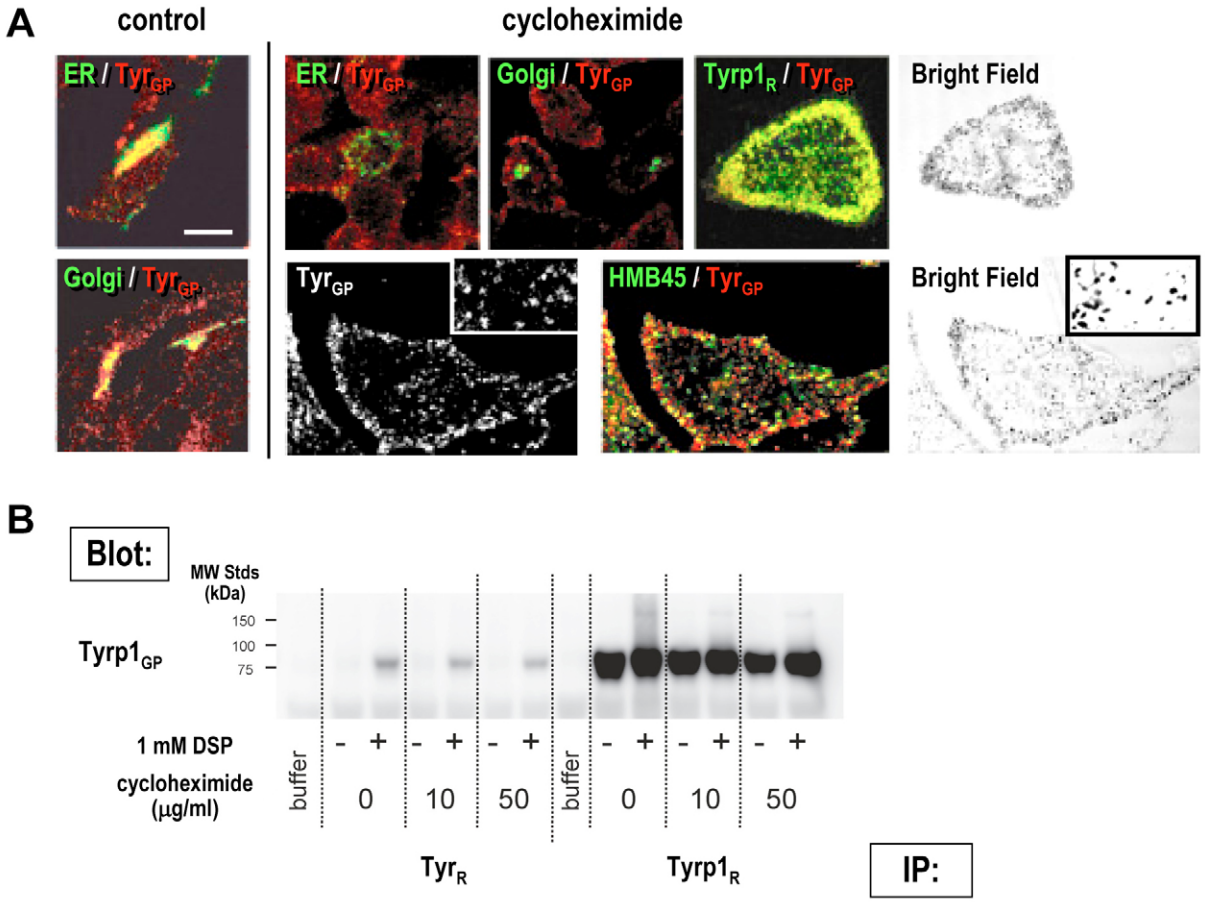

Fig. 4. Interaction of Tyr and Tyrp1 in cycloheximide-treated melanocytes. (A) Confocal images of melan-a melanocytes treated with or without cycloheximide. Melanocytes expressing YFP-ER or YFP-Golgi were treated with cycloheximide (10 μg/ml) for 10 hours, fixed and stained with the Tyr_{GP} antibody as described for Fig. 1. Melanocytes treated with cycloheximide were also double-stained with Tyr_R, $Tyrp1_{GP}$ or HMB45 as noted. Bright-field images are shown to view pigment granules in the cells. Bar, 10 μm. (B) Interaction of Tyr and Tyrp1 in cycloheximide-treated melan-a melanocytes was analyzed by chemical crosslinking with DSP, immunoprecipitation and immunoblotting, as described for Fig. 2.

Interaction among TRP family proteins via their EGF motifs has been previously proposed. Orlow and colleagues showed that the N-termini of Tyrp1 and Dct play important roles in the intermolecular association of TRP family proteins and in the stabilization of enzymatic activities (Manga et al., 2000). In this study, we were unable to detect any association of Tyr with Tyrp1 in melan-b melanocytes, which are mutant at the *Tyrp1* locus, even after treatment with DSP, showing that mutant Tyrp1 does not interact with Tyr. This suggests that the N-terminal region of Tyrp1, where the brown mutation results in an amino acid substitution of tyrosine for cysteine (C86Y), is critical for the physical interaction between Tyr and Tyrp1. However, this mutation in Tyrp1 also affects the processing and intracellular trafficking of the mutant protein, resulting in its quick degradation, which might reduce the time for it to interact with Tyr. Mutant Tyrp1 has been shown to form a complex with Tyr in the ER, which results in the proteasomal degradation of wild-type Tyr in Tyrp1 mutant melanocytes (Toyofuku et al., 2001). The correct processing of Tyr has been shown to be critical to avoid its degradation in proteasomes (Svedine et al., 2004). Thus, the mechanism of the intermolecular association of Tyr and Tyrp1 remains obscure and further studies are required.

Hebert's group recently reported the possibility of Tyr oligomerization in vitro as well as in vivo (Francis et al., 2003). They detected a high molecular mass complex containing Tyr in melanocytes using the chemical crosslinker, bismaleimidohexane (BMH), the molecular size of which corresponds to the Tyr dimer. There was no mention about the involvement of Tyrp1 as a component of that molecular complex but it seems unlikely that the Tyr-Tyrp1 complex would be detectable because BMH reacts with free thiols and its efficiency is low, whereas DSP used in our study reacts with free amine residues. Our results suggest that Tyr can form oligomers in melanocytes. Our two-dimensional SDS-PAGE results (Fig. 5) show that the amount of crosslinked Tyr, which migrates at ~150 kDa before cleavage, is relatively more abundant than that of crosslinked Tyrp1. Although immunoblotting analysis of two distinct proteins is not usually quantitative, we believe it is semiquantitative in this case because our primary antipeptide antisera against Tyr or Tyrp1 had similar titers against the antigenic peptides (Kobayashi et al., 1994a) and because all blots were stained using the same secondary antibody under identical conditions and were developed in parallel. That analysis suggests that Tyrp1 is about 10 times more abundant in melan-a melanocytes, but that

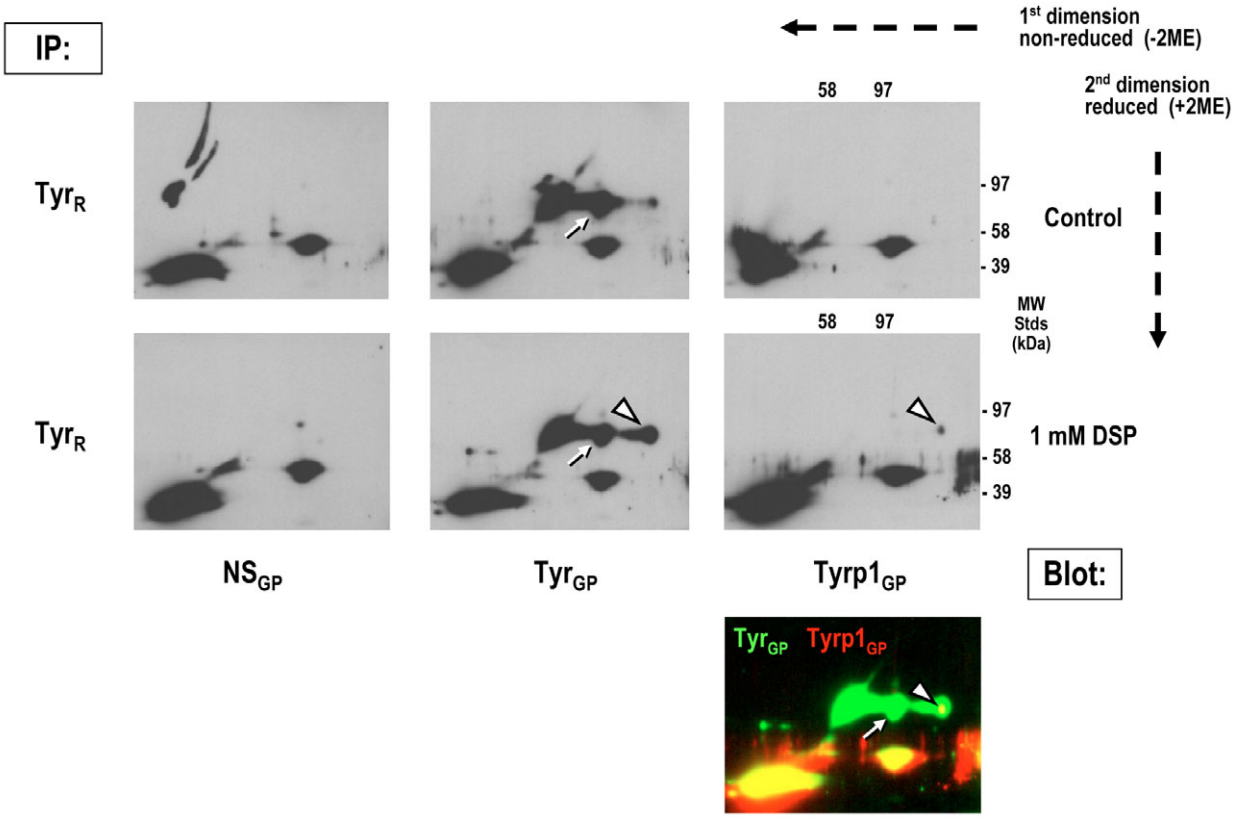

Fig. 5. Direct interaction of Tyr and Tyrp1. Tyr was immunoprecipitated from melan-a melanocytes pretreated with or without DSP, and was separated in the first dimension by SDS-PAGE under nonreducing conditions. Tyr and crosslinked proteins were cleaved and resolved in the second dimension by SDS-PAGE under reducing conditions. Blots of DSP-treated samples detected by anti-TyrGP (green) and Tyrp1 GP (red) were overlaid and are shown in the bottom panel. Arrowheads indicate Tyr or Tyrp1 detectable only when cells had been pretreated with DSP. Tyr spots marked with arrows were detected regardless of DSP treatment and probably represent Tyr that comigrated with the anti-Tyr IgG heavy chain on the first gel. There are also some non-specific spots visible at ~58 kDa. Representative results of two experiments are shown.

the efficiency of Tyrp1 crosslinking to Tyr is less than 10%. We cannot exclude the possibility that they form much larger molecular complexes for melanin synthesis, although only heterodimers of Tyr and Tyrp1 were detected by our two-dimensional SDS-PAGE analysis. These results suggest that Tyr interacts and forms a molecular complex with Tyrp1 and that Tyr is also likely to homodimerize because the crosslinked complex has the same size as a Tyr dimer (Fig. 3) and is much more abundant than Tyrp1. Francis et al. reported that the oligomerization of Tyr is impaired in Tyrp1-mutant melan-b cells, indicating that Tyrp1 may be involved in Tyr oligomerization (Francis et al., 2003). They suggested that Tyrp1 might interact with Tyr to facilitate the oligomerization.

In this study, we were unable to detect in vivo interactions of Tyr or Tyrp1 with Dct or with Silver, two other melanosomal proteins that are critical to pigmentation (Valencia et al., 2006). Interestingly, Dct also seems able to stabilize the activity of Tyr in vitro as does Tyrp1 (Hearing et al., 1992; Winder et al., 1994; Kobayashi et al., 1994c) and Dct forms a molecular complex with Tyr and Tyrp1 in vitro (Orlow et al., 1994). This suggested the possibility that Dct interacts with Tyr in vivo, a hypothesis not supported by our results. Dct is degraded more rapidly than Tyr or Tyrp1 in melanocytes (Kobayashi et al., 1998; Toyofuku et al., 2001), and levels of Dct are much lower in melanocytes compared with Tyrp1. Therefore, even if Dct associates with Tyr to a limited extent, we were unable to detect this after treatment with DSP, and it seems unlikely that Dct is crucial for Tyr stabilization in vivo. However, Tyrp1 might be crucial for the stabilization of Tyr in melanocytes because it is the most abundant glycoprotein (Tai et al., 1983; Vijayasaradhi and Houghton, 1991) and has a relatively longer life span than Tyrp2/Dct (Kobayashi et al., 1998; Toyofuku et al., 2001). Taken together, these data demonstrate that Tyrp1 interacts directly with Tyr in melanocytes and in melanosomes, which may regulate the stability and trafficking of those critical melanogenic enzymes and thus determines pigment biosynthesis.

Materials and Methods

Cells and culture conditions

Murine melanocyte lines derived from black (*Tyrp1*$^{+}$/*Tyrp1*$^{+}$, *Tyr*$^{+}$/*Tyr*$^{+}$), brown (*Tyrp1*^{b}/*Tyrp1*^{b}, *Tyr*$^{+}$/*Tyr*$^{+}$) and albino (*Tyrp1*$^{+}$/*Tyrp1*$^{+}$, *Tyr*^{c}/*Tyr*^{c}) non-agouti mice (melan-a, melan-b and melan-c cells, respectively), were generous gifts from Dorothy C. Bennett and Elena Sviderskaya (St George's Hospital, London, UK) (Bennett et al., 1987; Bennett et al., 1989; Bennett, 1991). Those cells were cultured as previously reported (Kobayashi et al., 1998).

Materials

The chemical crosslinkers, dithiobis(succinimidylpropionate) (DSP), disuccinimidyl suberate (DSS), bis[sulfosuccinimidyl] suberate (BS3) and 3,3′-dithiobis (sulfosuccinimidylpropionate) (DTSSP), ovalbumin and BCA protein assay reagents were purchased from Pierce Biotechnology (Rockford, IL). Nonidet P-40, EDTA-free protease inhibitor cocktail and FuGENE6 were obtained from Roche Diagnostics

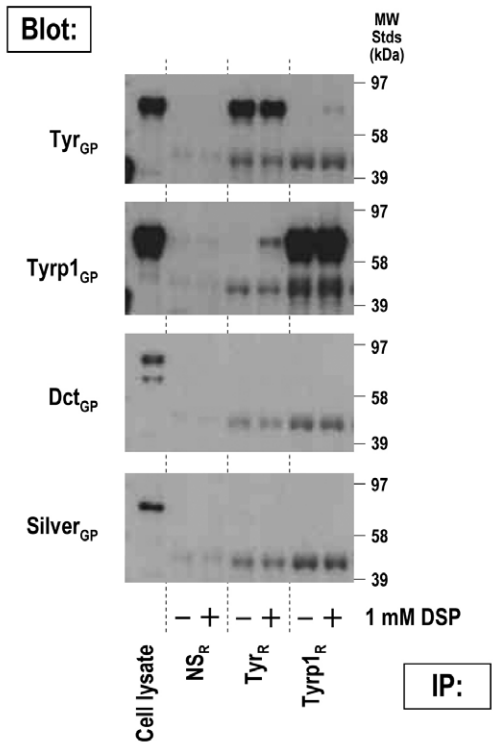

Fig. 6. Interaction of Tyr or Tyrp1 with other melanosomal proteins. Tyr or Tyrp1 was immunoprecipitated from melan-a melanocytes pretreated with or without 1 mM DSP as described for Fig. 2. Immunoblotting analysis was used to detect Tyr/Tyrp1 coprecipitates using Tyr$_{GP}$, Tyrp1$_{GP}$, Dct$_{GP}$ and Silver$_{GP}$ antibodies.

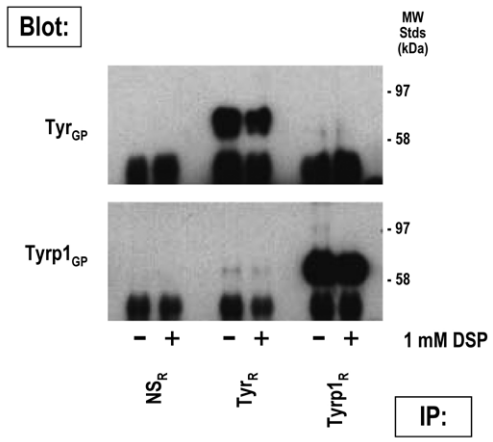

Fig. 7. Interaction of Tyr with Tyrp1 is disrupted in *Tyrp1b* mutant melanocytes. Tyr and Tyrp1 were immunoprecipitated from melan-b melanocytes by antibodies as noted at the bottom and then were examined by western blot analysis as detailed in Fig 2.

(Tokyo, Japan). Protein G Sepharose and enhanced chemiluminescence detection reagents were from GE Healthcare, (Buckinghamshire, UK). Gelatin and cycloheximide was purchased from Wako Chemical Co. (Osaka, Japan).

Antibodies

The rabbit antibodies used in this study, αPEP1, αPEP7, αPEP8 and αmSiN, were generated against synthetic peptides corresponding to the C- or N-termini of murine melanogenic proteins Tyrp1, Tyr, Dct and Silver, respectively. They specifically recognize each melanogenic protein as described previously (Jiménez et al., 1989; Tsukamoto et al., 1992a; Yasumoto et al., 2004). In this study, we refer to those antibodies by the name of the antigen recognized, with a subcrip 'R' to denote their origin from rabbits. The mouse monoclonal antibody HMB-45 was purchased from Enzo Life Sciences (Farmingdale, NY). HMB-45 recognizes a mature processed form of the Silver protein (Yasumoto et al., 2004; Theos et al., 2006) and specifically the fibrillar matrix of Stage II melanosomes (Kushimoto et al., 2001; Raposo et al., 2001).

For this study, we also generated antibodies in guinea pigs against the identical antigenic peptides used to generate the rabbit antisera noted above (namely, the C-termini of Tyrp1, Tyr and Dct, or the N-terminus of Silver). For immunization of guinea pigs, ovalbumin was used as a carrier protein for each antigenic peptide instead of keyhole limpet hemocyanin used for the rabbits. Analysis with an enzyme-linked immunosorbent assay showed that each guinea pig antibody had a similar high titer and specificity against the antigenic peptide as the rabbit antisera (data not shown). In western immunoblotting analysis of extracts of melan-a cells, the guinea pig antibodies reacted specifically with the proteins as expected and there were no apparent nonspecific bands. In this study, we refer to those antibodies by the name of the antigen recognized, with a subscript 'GP' to denote their origin from guinea pigs.

Immunopurification and immunodepletion of melanogenic proteins

Immunopurification of melanogenic proteins was done as described previously (Kobayashi et al., 1994a). Immunopurified proteins were further analyzed by western blot analysis as previously detailed (Kobayashi et al., 1995) using guinea pig peptide antisera. For immunodepletion, extracts of melan-a melanocytes (0.5 mg protein) were incubated with 20 μl rabbit antiserum, complexed with protein-G-Sepharose and sedimented. The resulting supernatants were used for western blot analysis.

Melanosome purification

Purification of melanosomes from murine melanocytes was carried out as described previously (Kobayashi et al., 1994a). All steps were performed at 4°C. Highly pigmented melan-a melanocytes were harvested, rinsed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS^-) and homogenized with 12 strokes of a Potter-Elvehjem grinder in homogenization buffer (0.25 M sucrose, 50 mM phosphate buffer, pH 6.8, 1 mM EDTA, protease inhibitor cocktail). Following centrifugation for 10 minutes at 1000 ***g***, each supernatant was centrifuged for 30 minutes at 10,000 ***g***. The pellets were resuspended in homogenization buffer and applied to a discontinuous gradient of 1.0-2.0 M sucrose and centrifuged for 1.5 hours at 100,000 ***g***. Melanosomes were collected at the 1.8-2.0 M and the 1.6-1.8 M interfaces. Purity of the melanosomal fractions isolated under these conditions has been described previously (Potterf et al., 1996).

Chemical crosslinking and immunoprecipitation

Crosslinking was performed according to the standard method described in the manufacturer's protocol. Briefly, harvested cells or purified melanosomes were resuspended in ice-cold PBS^- (2 ml) and combined with 20 μl DSP stock solution in dimethyl sulfoxide to a final concentration of 0-2 mM. In some experiments, other crosslinkers were used. After incubation of the reaction mixtures on ice for 20 minutes, the cells or the melanosomes were rinsed with 2 mM glycine in PBS^- and then with PBS^-, and solubilized on ice for 30 minutes in Nonidet P-40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, protease inhibitor cocktail). The extracts were centrifuged for 15 minutes at 10,000 ***g***, and the supernatants were kept on ice for BCA protein assay and immunoprecipitation.

The resulting supernatants (1 mg protein each) were precleared with 10 μl normal rabbit serum and 100 μl protein-G-Sepharose (GE Healthcare). The precleared extracts were combined with gelatin to a final concentration of 0.5 mg/ml gelatin to reduce the non-specific binding of melanogenic proteins to IgG and matrix, incubated with 5 μl rabbit antiserum for 1 hour at 4°C and were then complexed with 30 μl protein-G-Sepharose for 30 minutes at 4°C. The immune complexes were washed three times with Nonidet P-40/SDS buffer containing 0.5 mg/ml gelatin and then three times with Nonidet P-40/SDS buffer, eluted in SDS sample buffer containing 2-mercaptoethanol at 95°C for 3 minutes and separated by SDS gel electrophoresis (Laemmli, 1970), followed by western blot analysis as described previously (Kobayashi et al., 1994a; Kobayashi et al., 1998).

For 2D-PAGE analysis, the immune complexes were first eluted with non-reducing SDS sample buffer without any reducing agents and were resolved on SDS gels. After the first dimension separation, the lane was cut from the gel and boiled in sample buffer containing 5% 2-mercaptoethanol at 95°C for 3 minutes and then was separated on a SDS gel under reducing conditions (second dimension), and analyzed by western blot analysis.

Molecular sizes reported were estimated using standard protein mixtures from Sigma (St Louis, MO) and from Bio-Rad (Hercules, CA) to generate a mobility/size standard curve for each experiment. For western blot analysis, crosslinked and coprecipitated proteins were separated on PAGE gels that contained abundant amounts of eluted IgG, which frequently retarded their migration, and therefore, molecular sizes of crosslinked proteins reported are only estimates.

Expression of ER and Golgi markers

The ER- or Golgi-targeting EYFP vectors (pEYFP-ER Vector or pEYFP-Golgi Vector, BD Clontech, Mountain View, CA) were transiently expressed in melanocytes using FuGENE6, according to the manufacturer's instructions.

Immunofluorescence microscopy

Melanocytes grown on coverslips (Matsunami, No. 1) were fixed in 4% paraformaldehyde for 1 hour on ice, and were then quenched in ice-cold 10 mM glycine/PBS$^-$ for 20 minutes. After rinsing with ice-cold PBS$^-$, the cells were permeabilized with 0.05% Triton X-100 for 15 minutes on ice and blocked in blocking buffer (3% bovine serum albumin and 4% fetal bovine serum in PBS$^-$) overnight at 4°C. The cells were then immunostained with the primary antibody, followed by the appropriate Alexa Fluor 488- or Alexa Fluor 647-conjugated secondary antibodies (1:2000 dilution; Invitrogen, Carlsbad, CA). Fluorescence images were acquired using a laser-scanning confocal microscope (LSM510, Carl Zeiss) and processed with Adobe Photoshop software.

This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.K.),

References

Bennett, D. C. (1991). Colour genes, oncogenes and melanocyte differentiation. *J. Cell Sci.* **98**, 135-139.

Bennett, D. C., Cooper, P. J. and Hart, I. R. (1987). A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumor promoter for growth. *Int. J. Cancer* **39**, 414-418.

Bennett, D. C., Cooper, P. J., Dexter, T. H., Devlin, L. M., Heasman, J. and Nester, B. (1989). Cloned mouse melanocyte lines carrying germline mutations albino and brown: complementation in culture. *Development* **105**, 379-385.

Boissy, R. E., Zhao, H., Oetting, W. S., Austin, L. M., Wildenberg, S. C., Boissy, Y. L., Zhao, Y., Sturm, R. A., Hearing, V. J., King, R. A. et al. (1996). Mutation in and lack of expression of tyrosinase related protein 1 (TRP1) in melanocytes from an individual with brown oculocutaneous albinism: a new subtype of albinism classified as OCA3. *Am. J. Hum. Gen.* **58**, 1145-1156.

Costin, G. E., Valencia, J. C., Vieira, W. D., Lamoreux, M. L. and Hearing, V. J. (2003). Tyrosinase processing and intracellular trafficking is disrupted in mouse primary melanocytes carrying the uw mutation: a model for oculocutaneous albinism (OCA) type 4. *J. Cell Sci.* **116**, 3203-3212.

Francis, E., Wang, N., Parag, H., Halaban, R. and Hebert, D. N. (2003). Tyrosinase maturation and oligomerization in the endoplasmic reticulum require a melanocyte-specific factor. *J. Biol. Chem.* **278**, 25607-25617.

Hearing, V. J. (1999). Biochemical control of melanogenesis and melanosomal organization. *J. Invest. Dermatol. Symp. Proc.* **4**, 24-28.

Hearing, V. J. (2005). Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. *J. Dermatol. Sci.* **37**, 3-14.

Hearing, V. J. and Tsukamoto, K. (1991). Enzymatic control of pigmentation in mammals. *FASEB J.* **5**, 2902-2909.

Hearing, V. J., Tsukamoto, K., Urabe, K., Kameyama, K., Montague, P. M. and Jackson, I. J. (1992). Functional properties of cloned melanogenic proteins. *Pigment Cell Res.* **5**, 264-270.

Hirosaki, K., Yamashita, T., Wada, I., Jin, H. Y. and Jimbow, K. (2002). Tyrosinase and tyrosinase-related protein 1 require rab7 for their intracellular transport. *J. Invest. Dermatol.* **119**, 475-480.

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

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, TRP2, maps to and is mutated at the mouse *slaty* locus. *EMBO J.* **11**, 527-535.

Jiménez, M., Kameyama, K., Maloy, W. L., Tomita, Y. and Hearing, V. J. (1988). Mammalian tyrosinase: biosynthesis, processing and modulation by melanocyte stimulating hormone. *Proc. Natl. Acad. Sci. USA* **85**, 3830-3834.

Jiménez, M., Maloy, W. L. and Hearing, V. J. (1989). Specific identification of an authentic clone for mammalian tyrosinase. *J. Biol. Chem.* **264**, 3397-3403.

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 5,6-dihydroxyindole-2-carboxylic acid oxidase activity of tyrosinase-related protein-1 (TRP1). *J. Biol. Chem.* **269**, 17993-18001.

Jiménez-Cervantes, C., Martinez-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.

King, R. A., Pietsch, J., Fryer, J. P., Savage, S., Brott, M. J., Russell-Eggitt, I., Summers, C. G. and Oetting, W. S. (2003). Tyrosinase gene mutations in oculocutaneous albinism 1 (OCA1): definition of the phenotype. *Hum. Genet.* **113**, 502-513.

Kobayashi, T., Urabe, K., Orlow, S. J., Higashi, K., Imokawa, G., Kwon, B. S., Potterf, S. B. and Hearing, V. J. (1994a). The pmel17/*silver* locus protein: characterization and investigation of its role in mammalian melanogenesis. *J. Biol. Chem.* **269**, 29198-29205.

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

Kobayashi, T., Urabe, K., Winder, A. J., Tsukamoto, K., Brewington, T., Imokawa, G., Potterf, S. B. and Hearing, V. J. (1994c). The DHICA oxidase activity of TRP1 and interactions with other melanogenic enzymes. *Pigment Cell Res.* **7**, 227-234.

Kobayashi, T., Vieira, W. D., Potterf, S. B., Sakai, C., Imokawa, G. and Hearing, V. J. (1995). Modulation of melanogenic protein expression during the switch from eu- to pheomelanogenesis. *J. Cell. Sci.* **108**, 2301-2309.

Kobayashi, T., Imokawa, G., Bennett, D. C. and Hearing, V. J. (1998). Tyrosinase stabilization by Tyrp1 (the *brown* locus protein). *J. Biol. Chem.* **273**, 31801-31805.

Kushimoto, T., Basrur, V., Valencia, J. C., Matsunaga, J., Vieira, W. D., Muller, J., Appella, E. and Hearing, V. J. (2001). A new model for melanosome biogenesis based on the purification and mapping of early melanosomes. *Proc. Natl. Acad. Sci. USA* **98**, 10698-10703.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Manga, P., Sato, K., Ye, L., Beermann, F., Lamoreux, M. L. and Orlow, S. J. (2000). Mutational analysis of the modulation of tyrosinase by tyrosinase-related proteins 1 and 2 in vitro. *Pigment Cell Res.* **13**, 364-374.

Marks, M. S. and Seabra, M. C. (2001). The melanosome: membrane dynamics in black and white. *Nat. Rev. Mol. Cell Biol.* **2**, 1-11.

Orlow, S. J., Zhou, B. K., Chakraborty, A. K., Drucker, M., Pifko-Hirst, S. and Pawelek, J. M. (1994). High-molecular-weight forms of tyrosinase and the tyrosinase-related proteins: evidence for a melanogenic complex. *J. Invest. Dermatol.* **103**, 196-201.

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

Potterf, S. B., Muller, J., Bernardini, I., Tietze, F., Kobayashi, T., Hearing, V. J. and Gahl, W. A. (1996). Characterization of a melanosomal transport system in murine melanocytes mediating entry of the melanogenic substrate tyrosine. *J. Biol. Chem.* **271**, 4002-4008.

Raposo, G., Tenza, D., Murphy, D. M., Berson, J. F. and Marks, M. S. (2001). Distinct protein sorting and localization to premelanosomes, melanosomes and lysosomes in pigmented melanocytic cells. *J. Cell Biol.* **152**, 809-823.

Seiji, M., Shimao, K., Birbeck, M. S. C. and Fitzpatrick, T. B. (1963). Subcellular localization of melanin biosynthesis. *Ann. N. Y. Acad. Sci.* **100**, 497-533.

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.

Svedine, S., Wang, T., Halaban, R. and Hebert, D. N. (2004). Carbohydrates act as sorting determinants in ER-associated degradation of tyrosinase. *J. Cell Sci.* **117**, 2937-2949.

Tai, T., Eisinger, M., Ogata, S. and Lloyd, K. O. (1983). Glycoproteins as differentiation markers in human malignant melanoma and melanocytes. *Cancer Res.* **43**, 2773-2779.

Theos, A. C., Berson, J. F., Theos, S. C., Herman, K. E., Harper, D. C., Tenza, D., Sviderskaya, E. V., Lamoreux, M. L., Bennett, D. C., Raposo, G. et al. (2006). Dual loss of ER export and endocytic signals with altered melanosome morphology in the *silver* mutation of Pmel17. *Mol. Biol. Cell* **17**, 3598-3612.

Toyofuku, K., Wada, I., Valencia, J. C., Kushimoto, T., Ferrans, V. J. and Hearing, V. J. (2001). Oculocutaneous albinism (OCA) types 1 and 3 are ER retention diseases: Mutations in tyrosinase and/or Tyrp1 influence the maturation, degradation of calnexin association of the other. *FASEB J.* **15**, 2149-2161.

Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. M. and Hearing, V. J. (1992a). A second tyrosinase-related protein, TRP2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J.* **11**, 519-526.

Tsukamoto, K., Palumbo, A., d'Ischia, M., Hearing, V. J. and Prota, G. (1992b). 5,6-Dihydroxyindole-2-carboxylic acid is incorporated into mammalian melanin. *Biochem. J.* **286**, 491-495.

Valencia, J. C., Watabe, H., Chi, A., Rouzaud, F., Chen, K. G., Vieira, W. D., Takahashi, K., Yamaguchi, Y., Berens, W., Nagashima, K. et al. (2006). Sorting of Pmel17 to melanocytes through the plasma membrane by AP1 and AP2: evidence for the polarized nature of melanocytes. *J. Cell Sci.* **119**, 1080-1091.

Vijayasaradhi, S. and Houghton, A. N. (1991). Purification of an autoantigenic 75-kDa human melanosomal glycoprotein. *Int. J. Cancer* **47**, 298-303.

Wang, N. and Hebert, D. N. (2006). Tyrosinase maturation through the mammalian secretory pathway: bringing color to life. *Pigment Cell Res.* **19**, 3-18.

Winder, A. J., Kobayashi, T., Tsukamoto, K., Urabe, K., Aroca, P., Kameyama, K. and Hearing, V. J. (1994). The tyrosinase gene family: Interactions of melanogenic proteins to regulate melanogenesis. *Cell. Mol. Biol. Res.* **40**, 613-626.

Wu, H. and Park, H. Y. (2003). Protein kinase C-beta-mediated complex formation between tyrosinase and TRP-1. *Biochem. Biophys. Res. Commun.* **311**, 948-953.

Yasumoto, K., Watabe, H., Valencia, J. C., Kushimoto, T., Kobayashi, T., Appella, E. and Hearing, V. J. (2004). Epitope mapping of the melanosomal matrix protein gp100 (PMEL17): Rapid processing in the endoplasmic reticulum and glycosylation in the early Golgi compartment. *J. Biol. Chem.* **279**, 28330-28338.

Zhao, H., Eling, D. J., Medrano, E. E. and Boissy, R. E. (1996). Retroviral infection with human tyrosinase-related protein-1 (TRP1) cDNA upregulates tyrosinase activity and melanin synthesis in a TRP-1 deficient melanoma cell line. *J. Invest. Dermatol.* **106**, 744-752.