TYRP1 in melanosomes is not correlated to the anti-cytotoxicity against tyrosinase. These results indicate that the anti-cytotoxic activity of TYRP1 against tyrosinase seems to be independent of its localization in melanosomes, or interaction with tyrosinase and melanogenic activity. Since the mutation sites that affected the anti-cytotoxic activity of TYRP1 (S38C, C110Y, S354C and D517Y) were scattered on the molecule from 38^{th} to 517^{th} codon (Fig. 1), it is suggested that the biologic activity might be associated with the whole protein conformation.

Johnson & Jackson [33] reported that a single missense mutation of TYRP1 results in an increased risk of cytotoxicity in mouse melanocytes. Similarly in the *in vitro* culture of murine melanocytes, TYRP1 mutations resulted in the attenuation of cell proliferation rates and DNA synthesis [34, 35] recently reported that murine TYRP1 stabilizes tyrosinase but decreases its activity, while DCT increases tyrosinase activity by stabilizing the protein. The compartmentalization of tyrosinase may result in localization of the melanin intermediates that are cytotoxic in the melanosomes [3]. Previous reports showed that tyrosinase with mutations in copper-binding domains were unable to interact with calnexin or calreticulin and, thus, were not transported to melanosomes [36, 37]. Thus, proper transport of TYRP1 into melanosomes seems to be important for its melanogenic function. It is unclear how H192L mutant exerts its function to suppress tyrosinase-mediated cell death, since it might lose its ability to interact with calnexin and/or calreticulin and consequently its capacity to be transported to melanosomes. The result obtained by H192L and H377L suggests that suppressive functions of TYRP1 against tyrosinase-mediated cell death might not

require appropriate transport to melanosomes or interaction with tyrosinase. However, it is possible that H192L might be transported to melanosomes but none the less lose its melanogenic function.

From these results, it appears that the two biologic abilities of TYRP1, namely melanogenic activity and suppression of tyrosinase-mediated cell death are functionally separate from each other. It is, therefore, concluded that the functional domains of TYRP1, which play an additive role in tyrosinase activity and melanin production, are localized in different sites of the molecule for the inhibition in tyrosinase-mediated cytotoxicity. Further analysis should be performed to elucidate the molecular basis of the anti-cytotoxic ability of TYRP1.

Acknowledgment

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Figure Legends

Figure 1. Schematic representation of TYRP1 mutants aligned with wtTYRP1. □ indicates the position of an amino acid substitution of each missense mutant. dl137/267 is predicted to express a polypeptide with amino acid substitution at 137th codon and deletion from 138th through 267th codon. dl260/322 deletes amino acids from Ile 260 through Pro322. dl260 deletes C-terminal region beyond Ile260. Amino acid changes of missense mutations S38C, C110Y, H192L, S354C, H377L and D517Y are listed in Table1.

Figure 2. Detection of TYRP1 mutants mRNA by RT-PCR. The mRNA was purified from SK-mel-24 cells inoculated with recombinant adenovirus at the moi of 20 pfu/cell and cultured for 24 hrs. PCR was carried out by using cDNA as a substrate as described in the materials and methods. One tenth of the amplified products was electrophoresed on 1% agarose gels. (A) PCR products by the primer set TYRP1-4 and TYRP1-343. (B) PCR products by TYRP1-616 and TYRP1-1555. (C) PCR products by GA1 and GA2 (controls).

Figure 3. Mutant TYRP1 proteins detected by Western blotting. Cells were infected with Ad-TYRP1 or Ad-TYRP1 mutants (20 pfu/cell) and cultured for 60 hrs. Cellular lysates containing 5.0 μg protein were electrophoresed in 5-20% SDS-PAGE, transferred to a nitrocellulose membrane and reacted with the polyclonal antibody as described in the Materials and Methods.

Figure 4. Melanin production by co-expression of TYRP1 mutants with tyrosinase. After SK-mel-24 cells were infected with recombinant adenoviruses for 60 hrs, cells were collected and processed for measurement of melanin

content. Means±SD was determined from three dishes per infection. All melanin productions in co-infected samples except for H192L, H377L, dl137/267, dl260/322 and dl260 were significantly different ($*p<0.05$) from samples infected with tyrosinase alone.

Figure 5. Growth of cells infected with Ad-TYR plus Ad-TYRP1 or Ad-TYRP1 mutants. After 2 X 10^5 of SK-mel-24 cells were seeded in 6 cm dishes and cultured for 24 hrs, cells were infected with Ad-TYR or Ad-TYR plus Ad-TYRP1 or Ad-TYR plus Ad-TYRP1 mutant at a total moi of 50 pfu/cell and numbers of cells were counted on the 7th day after infection. Cells were detached and the numbers of viable cells were counted by a hemocytometer. Means±SD was determined from three dishes per infection. All cell numbers in co-infected samples with Lac Z, wtTYRP1 and H192L were significantly different ($*p<0.05$) from samples infected with tyrosinase alone.

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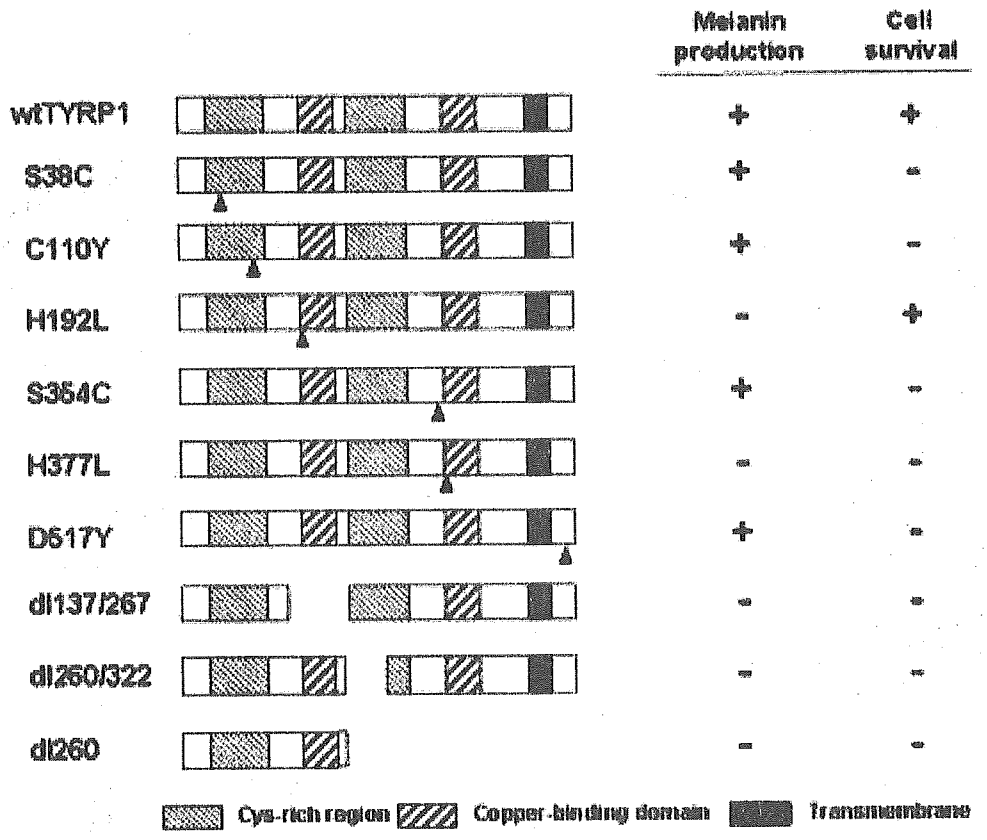

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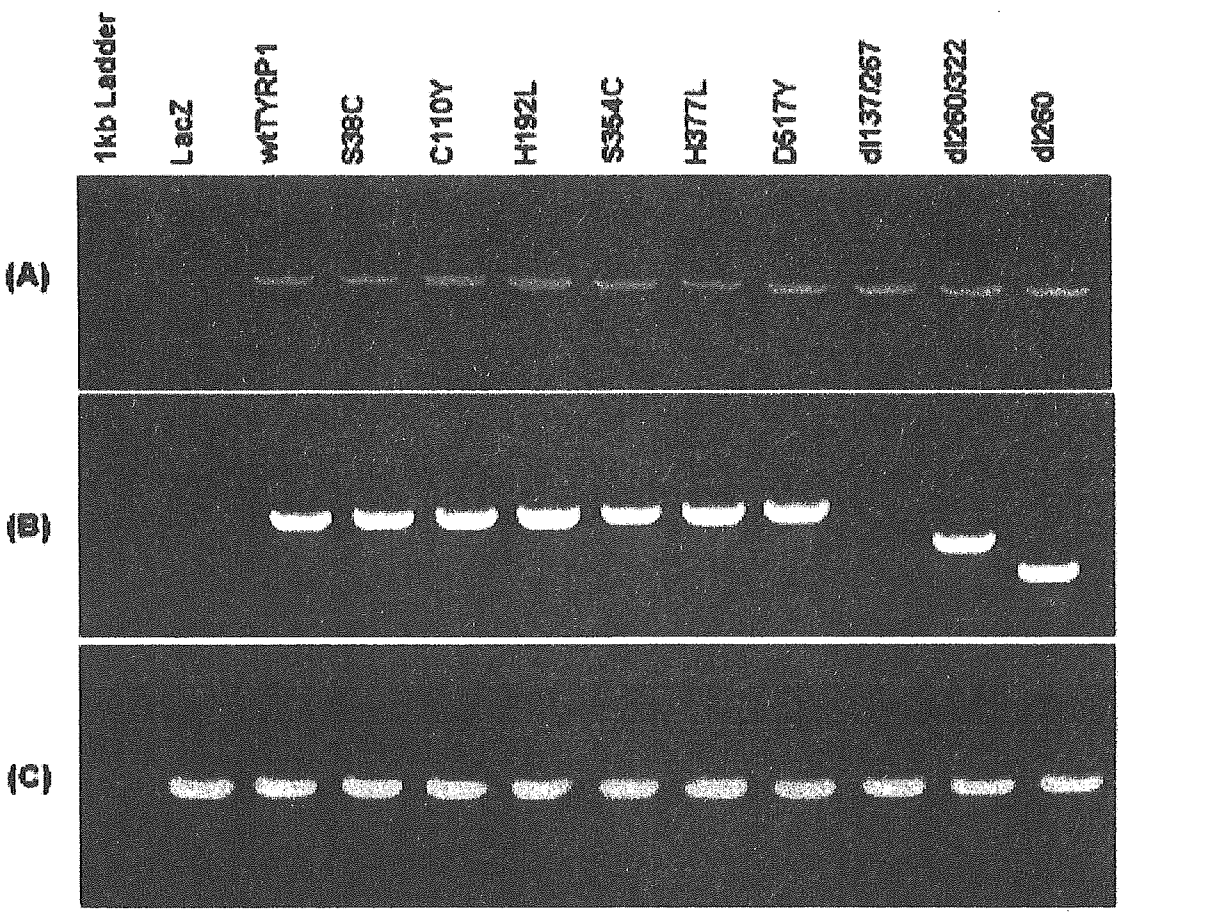

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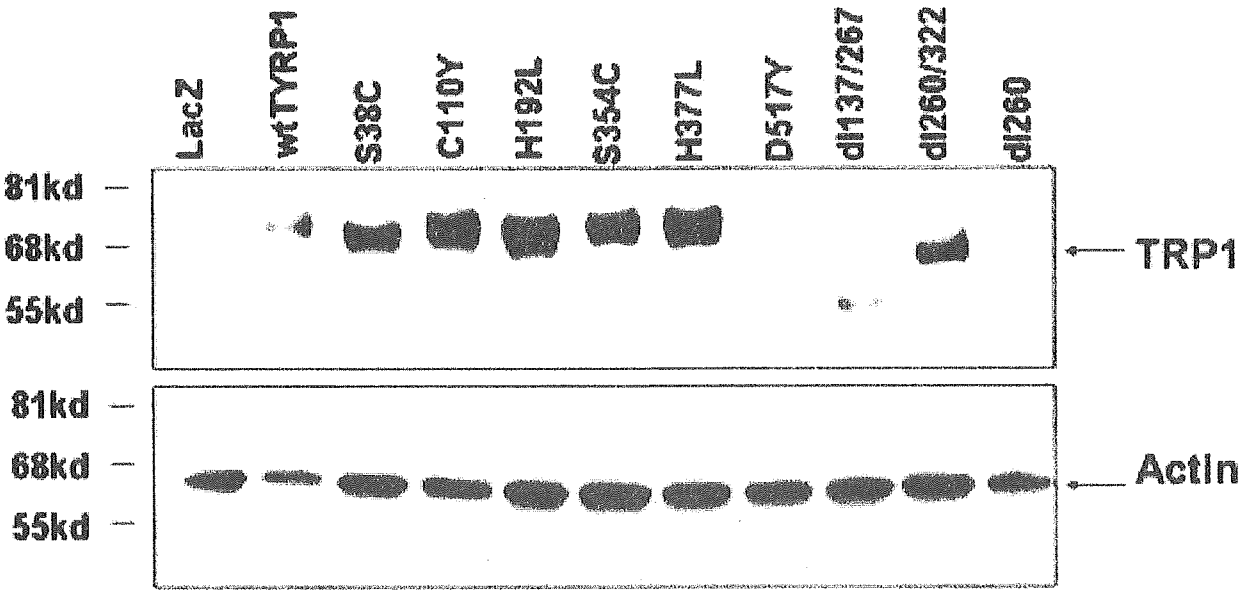

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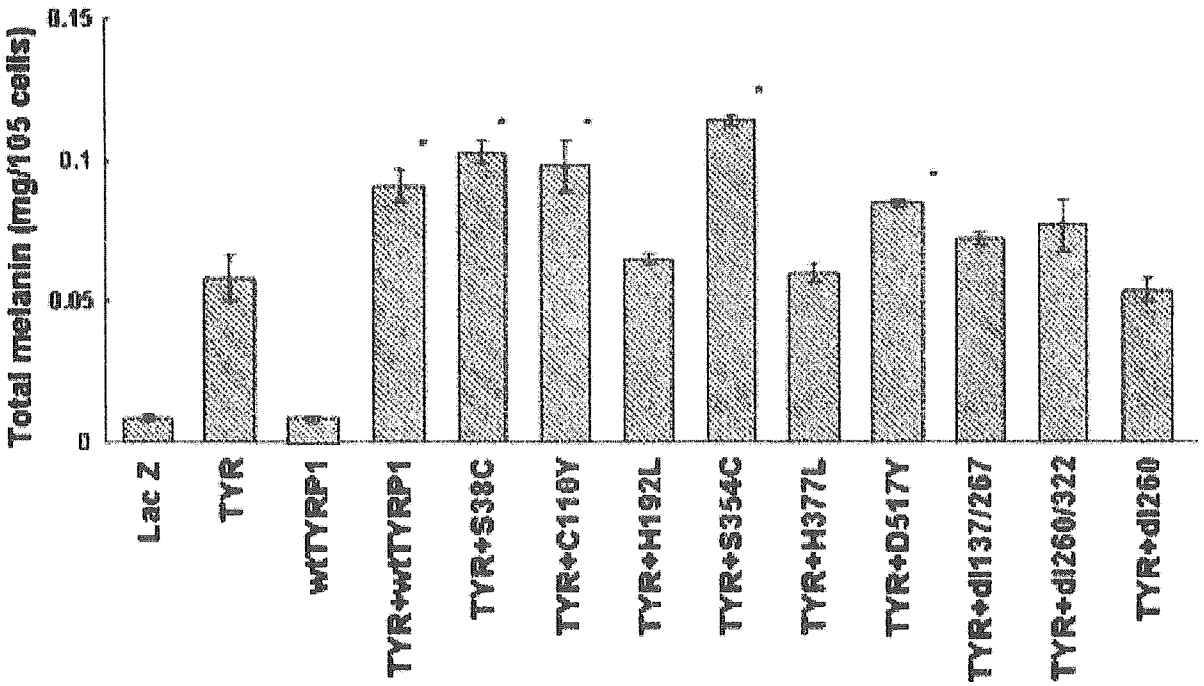

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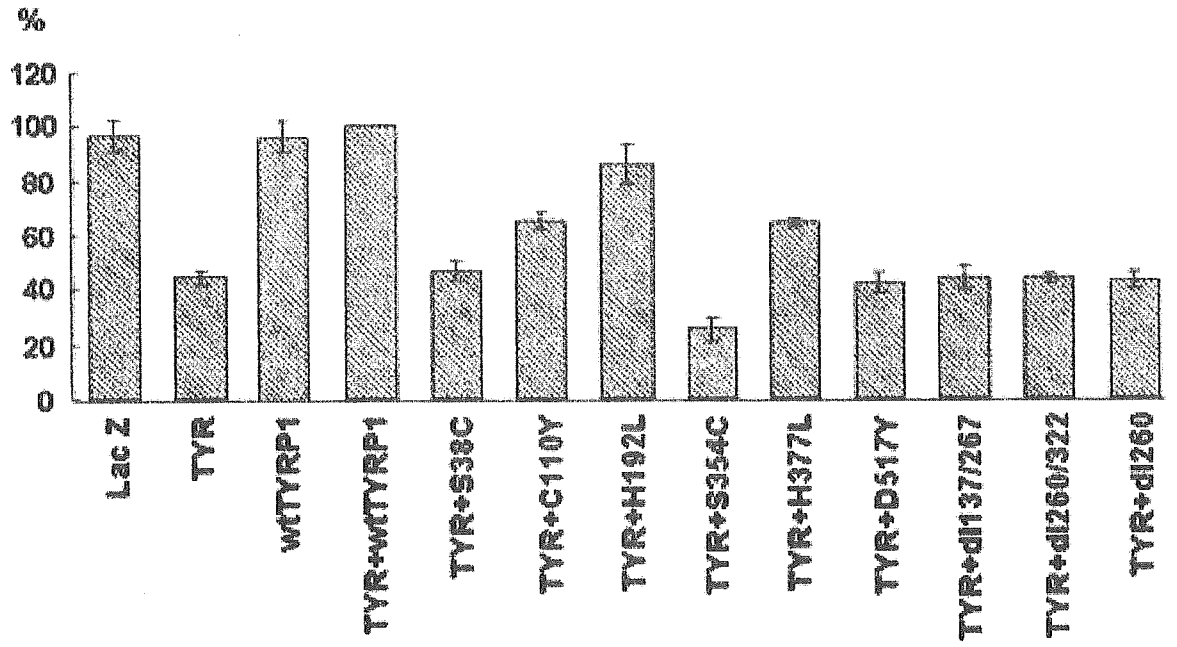

Table
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Table1.

Mutant	Amino acid changes
S38C	Ser^{38} to Cys^{38}
C110Y	Cys^{110} to Tyr^{110}
H192L	His^{192} to Leu^{192}
S354C	Ser^{354} to Cys^{354}
H377L	His^{377} to Leu^{377}
D517Y	Asp^{517} to Tyr^{517}
dl137/267	Ser^{137} to Arg^{137}, deletion from Lys^{138} to Gly^{267}
dl260/322	Deletion from Ile^{260} to Pro^{322}
dl260	Deletion beyond Ile^{260}

Dominant negative Rab7 induces vesicular transport unique to Tyrp1 compared to other melanosomal proteins, tyrosinase and gp100 in melanogenesis cascade.

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Key words: endosome, vesicular transport, Tyrp1, tyrosinase, gp100, proteasome, melanogenesis

Abbreviations: AP, adaptor protein; EEA1, early endosome antigen-1; mAb, monoclonal

antibody; MPR, mannose phosphate receptor; MVB, Multivesicular body; Tyrp, tyrosinase-related protein; TGN, *trans*-Golgi network; WT, wild-type

Abstract

We have previously shown that Rab7, a small GTP-binding protein, is involved in melanogenesis cascade through participation in the transport of melanosomal proteins. This study further examined the biological role of Rab7 and compared the vesicular transport of tyrosinase, tyrosinase-related protein-1 (Tyrp1) and gp100 in immortal murine melanocytes (melan-a) transfected with plasmids carrying wild-type Rab7 (Rab7WT) or dominant negative Rab7 (Rab7N125I).

Specifically, the distribution of melanosomal proteins was analyzed in the presence or absence of Rab7 function by immunofluorescent staining and confocal laser scanning microscopy. In Rab7WT-expressing melan-a cells, tyrosinase, Tyrp1 and gp100 were detected throughout the cytoplasm. In Rab7N125I-expressing cells, however, Tyrp1 was gradually eliminated by 48 h after the transfection, while TYR and gp100 were not.

The elimination of Tyrp1 was also observed in the cells expressing another dominant negative mutant and a constitutively-active mutant, Rab7T22N and Rab7Q67L, respectively, and was rescued by proteasomal inhibitor, not by lysosomal inhibitor.

These results suggest that the transport of Tyrp1 from Golgi complex to melanosomes requires Rab7 function that the inhibition of Rab7 leads to Tyrp1 elimination due to proteasomal activity, and that the vesicular transport of Tyrp1 is different from that of tyrosinase and gp100.