Coexpression of Wild-Type Tyrosinase Enhances Maturation of Temperature-Sensitive Tyrosinase Mutants

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Tyrosinase is a type I membrane glycoprotein whose activity is essential for melanin synthesis. Loss of function mutations in tyrosinase is the cause of oculocutaneous albinism 1. In the milder oculocutaneous albinism 1B form in which mutant proteins retain residual activity, the severity of albinism depends on the type of mutations expressed in the melanocyte. In this study, we show that coexpression of wild-type protein with temperature-sensitive tyrosinase mutants corrects the mutant conformation defect in an activity-dependent manner. Exit from the endoplasmic reticulum and complex carbohydrate processing in the Golgi was promoted when temperature-sensitive tyrosinase mutants were ectopically expressed in host melanocytes carrying wild-type protein even at the nonpermissive temperature. Incubation of transfected melanocytes with DOPA (the cofactor and substrate for tyrosinase), or tyrosine (the substrate), further enhanced processing of ectopic mutant proteins. The analysis of glycosylation-deficient mutants revealed regions in tyrosinase with high, low, and intermediate dependency on glycans for maturation. We concluded that the presence of tyrosinase activity enhances the maturation of temperature-sensitive and glycosylation-deficient forms of tyrosinase. The results may explain the variation in pigmentation and the development of pigment later in life in patients carrying different mutant alleles of oculocutaneous albinism 1B. ***Key words: albinism/endoplasmic reticulum/glycosylation and temperature-sensitive mutants/protein folding/quality control. J Invest Dermatol 119:481–488, 2002***

Pigmentation is a multistep process dependent on the functional integrity of tyrosinase (TYR, monophenol monooxygenase, EC 1.14.18.1), the rate-limiting activity in melanin synthesis. The enzyme catalyzes the oxidation of monohydric and dihydric phenols (catechols) to their corresponding quinone (Lerner *et al*, 1949). Mutations in TYR are the cause of classic type I oculocutaneous albinism (OCA1, OMIM 203100), an autosomal recessive genetic disorder characterized by melanin deficiency in melanocytes resulting in complete absence of pigmentation in the skin, hair, and eyes (King, 1998; Oetting and King, 1999). The mutations identified in *TYR* fall under two main functional groups: those that cause complete loss of tyrosinase activity associated with severe albinism (OCA1A), and those preserving residual activity (5%–10% of wild-type protein) conferring a slightly less severe albino phenotype (OCA1B) (reviewed in King, 1998; Oetting, 2000; see also Albinism Database at URL http://www.cbc.umn.edu/tad).

Tyrosinase is a type I membrane glycoprotein consisting of 529 amino acids (including the N-terminal signal peptide of 18 amino acids, accession number XM_027058) with a polypeptide backbone of ≈60 kDa. It is synthesized on endoplasmic reticulum (ER) bound ribosomes and the cotranslational transfer of six or seven N-linked oligosaccharide chains increases its molecular weight to ≈70 kDa (Halaban *et al*, 1983, 1984, 1997; Újvári *et al*, 2001; see **Fig 7**). Under normal conditions, complex sugar modifications in the Golgi apparatus causes a further increase to ≈80 kDa, the size of the mature wild-type isoform that is then transported to the melanosome, the site of melanin synthesis (Halaban *et al*, 1983, 1997).

The exit of tyrosinase from the ER and its traffic through the secretory pathway is tightly regulated depending on proper folding and sorting. Misfolded proteins, such as mutant TYR expressed in albino melanocytes, are retained in the ER as the immature 70 kDa glycoform bound to the ER chaperones calnexin and calreticulin (Berson *et al*, 2000; Halaban *et al*, 2000; Toyofuku *et al*, 2001). In addition, genetic analysis of inherited hypopigmentation disorders demonstrated that mutations in other genes modify tyrosinase stability and subcellular localization and/or function. For example, individuals with OCA2 (OMIM 203200) suffer loss-of-function mutations in the p-protein (Gardner *et al*, 1992; Rinchik *et al*, 1993; Lee *et al*, 1994; Oetting *et al*, 1998), which causes inactivation of tyrosine within the mutant cells, disruption of melanosomal structure, and misrouting of tyrosinase to other sites including the cell membrane (Potterf *et al*, 1998; Orlow and Brilliant, 1999; Puri *et al*, 2000). The albino phenotype of OCA2 is less severe than that of OCA1A, but is similar to OCA1B in that some affected individuals accumulate pigment later in life. The clinical manifestations of OCA1B and OCA2 are often indistinguishable, indicating that suppression of tyrosinase function can lead to a similar phenotype (King, 1998; Passmore *et al*, 1999).

We have recently shown that exit of wild-type tyrosinase from the ER to the Golgi is facilitated by its cofactor supplied in the

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Abbreviations: Endo H, endoglycosidase H; ER, endoplasmic reticulum; OCA1, oculocutaneous albinism 1; TYR, human tyrosinase; Tyr, mouse tyrosinase; WGA, wheat germ agglutinin.

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medium or generated by its own enzymatic activity (Halaban *et al*, 2001). Failure to exit the ER is conferred not only on loss-of-function mutant proteins, but also on wild-type tyrosinase with diminished activity due to tumor-induced metabolic changes in its immediate milieu, as in the case of melanoma cells (Halaban *et al*, 1997, 2001). In tyrosinase-positive amelanotic melanoma cells inactivated tyrosinase accumulates in the ER and is then subsequently targeted to cytosolic proteasome for degradation (Halaban *et al*, 1997, 2001). Tyrosinase maturation and pigmentation were restored in melanoma cells by treatments with tyrosine, DOPA, or alkalinization, conditions that induce enzymatic activity within these cells (Halaban *et al*, 2001, 2002).

We therefore proposed that tyrosinase oxidation products enhance proper folding of wild-type TYR and raised the possibility that mutant OCA1B proteins with residual activity can also be rescued by increasing the levels of tyrosinase reaction products. This concept was explored by the analysis of a temperature-sensitive variant and mutant proteins destabilized by the elimination of specific glycans. The results show that processing of the temperature-sensitive glycoforms was restored by coexpression with wild-type tyrosinase in an activity-dependent manner.

MATERIALS AND METHODS

Cell cultures Immortalized mouse melanocytes established from a black B10BR mouse (Tamura *et al*, 1987) termed wild-type, albino melan c melanocytes from a Balb-c mouse (Bennett *et al*, 1989) homozygous for the *c*-locus (*Tyr*) mutation C85S (Kwon *et al*, 1989; Jackson and Bennett, 1990), termed albino, COS7 cells, and human metastatic melanoma cells 501 mel were used for transfection. Normal mouse melanocytes were cultured in Ham's F-10 medium supplemented with glutamine (2 mM), penicillin–streptomycin (100 U per ml), 7% horse serum (all from Gibco Life Technologies, Rockville, MD), and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, 85 nM) (Sigma-Aldrich, St. Louis, MO) at 37°C in a 5% CO_2 humidified incubator. In some experiments the Ham's F-10 medium, which contains 10 μM tyrosine, was supplemented with tyrosine to a final concentration of 200 μM or 400 μM, as indicated, and/or with freshly prepared DOPA (50 μM). COS7 cells were grown in RPMI (Gibco) and the melanoma cells in Ham's F-10 medium, both supplemented with 7% fetal bovine serum (Gemini Bio-Products, Woodland, CA).

Construction of plasmids encoding wild-type and mutant tyrosinase proteins The plasmid pcTYR carrying the human tyrosinase gene was a gift from Dr. R. Spritz (Denver, CO). To generate FLAG-tagged tyrosinase proteins, human tyrosinase encoding the wild-type or mutant protein carrying the R402Q variant (Tripathi *et al*, 1991) were excised from pCMV/TYR-GFP plasmids (Halaban *et al*, 2000) and ligated into the HindIII/KpnI cloning sites of p3XFLAG-CMV-14 expression vector (Sigma). The QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) was used to generate single-site glycosylation deletion mutations, in which the consensus N-linked glycosylation sites Asn-X-Thr/Ser in TYR-FLAG were eliminated or modified in the cDNA by changing threonine or serine to an alanine, except for Thr373, which was changed to lysine to mimic the albino mutation T373K as described previously (Újvári *et al*, 2001). The site at Asn290 was also modified by exchanging the proline at position 293 to alanine (P293A) (Újvári *et al*, 2001). In all cases, DNA sequencing of the tyrosinase gene verified the inserted mutations. DOTAP-encapsulated plasmid DNAs (20 μg each) were transfected into wild-type (B10BR) or albino melan c mouse melanocytes carrying the loss-of-function Tyr(C85S) mutation, following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Melanocytes were subjected to selection in 0.4 mg per ml G418 starting 4–5 d after transfection.

Western blotting and precipitation Melanocytes were lyzed in CHAPS buffer (2% CHAPS in 50 mM HEPES and 200 mM NaCl, pH 7.5) containing a cocktail of protease inhibitors ("Complete" EDTA-free, Boehringer Mannheim). Western blotting was performed with whole cell lysates (40 μg protein per lane), FLAG-immunoprecipitated products, or bead-bound wheat germ agglutinin (WGA, Sigma) affinity purified glycoproteins.

Ectopic chimeric human tyrosinase was detected with anti-FLAG monoclonal antibody (M2, Sigma) or T311 monoclonal antibody recognizing human but not mouse tyrosinase (Chen *et al*, 1995), and host mouse tyrosinase with goat M-19 polyclonal antibodies raised against the C-terminal peptide of mouse tyrosinase (Santa Cruz Biotechnology, CA). Antigen–antibody complexes were detected by enhanced chemiluminescence (ECL, NEN Life Science Products, Boston, MA) using the appropriate secondary antibody linked to horseradish peroxidase. Densities of tyrosinase bands on the X-ray films were estimated using an NIH image analyzer.

Tyrosinase activity Tyrosinase assays were performed with L-tyrosine-[3,5-^{3}H] (Perkin-Elmer Life Sciences) as a substrate (Pomerantz, 1976; Halaban and Lerner, 1977; Halaban *et al*, 1988). Briefly, TYR-FLAG chimeric proteins were precipitated from 2% CHAPS cell lysates (≈250 μg per precipitate) with 2 μg of anti-FLAG M2 monoclonal antibody and protein G-Sepharose beads (GammaBind G, Amersham Pharmacia Biotech, Sweden) by rotating for 2 h in the cold. The bead-bound immune complexes were then washed successively three times with lysis buffer, and bead-bound immunoprecipitates (200 μl final volume), were incubated with 50 μM L-tyrosine, 1 μCi per assay L-tyrosine-[3,5-^{3}H], and 50 μM L-DOPA, for 30 min at 37°C. Reactions were stopped with 200 μl solution of 10% activated charcoal in 0.1 M citric acid (wt/vol), the charcoal slurries were passed through Dowex columns (350 μl), and radioactivity of the eluate in scintillation fluid was measured with a scintillation counter. Alternatively, whole cell lysates (100 μg protein per assay) derived from TYR-FLAG transfected COS7 cells were used directly to assay tyrosinase activity. Parallel reactions with extracts from mouse albino melanocytes devoid of tyrosinase activity and nontransfected COS7 cells were used as negative controls (blanks), and wild-type mouse melanocytes as a positive control. The assays were carried out in duplicate or triplicate reactions and data are given as average cpm with blanks subtracted. Standard deviation between duplicates did not exceed 10% of total counts. All assays were repeated once.

Carbohydrate cleavage Cell lysates (≈250 μg protein) in 100 μl CHAPS lysis buffer were incubated with 50 μl of bead-bound WGA for 2 h in the cold, under constant rotation. After washing twice with CHAPS buffer and once with phosphate-buffered saline, bead-bound glycoproteins were digested with endoglycosidase H (Endo H), according to the manufacturer's instruction (Boehringer Mannheim). Reaction products were subjected to Western blotting with anti-FLAG or antityrosinase monoclonal antibodies.

RESULTS

TYR-FLAG chimera displays normal processing and possesses tyrosinase activity The integrity of a TYR-FLAG chimera was assessed by analyzing its sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) migration pattern, carbohydrate modification, enzymatic activity, and its ability to induce the synthesis of melanin. Expression in wild-type mouse melanocytes produced the 80–90 kDa chimeric protein that was largely resistant to Endo H digestion (**Fig 1*A***, *lanes 1, 2*). The small decrease in the electrophoretic mobility of the mature protein after Endo H digestion is similar to that observed in native human tyrosinase (Halaban *et al*, 2001), and is probably due to digestion of a portion of the seven glycans that escaped complex sugar processing in the Golgi (Újvári *et al*, 2001).

In the heterologous cellular system COS7 cells, TYR-FLAG was slightly smaller in size compared to that synthesized in wild-type mouse melanocytes (**Fig 1*A*,** compare *lanes 1–3*), possibly due to incomplete complex carbohydrate processing. In addition, a portion of the protein was digested by Endo H to the deglycosylated form (**Fig 1*A*,** *lane 4*, band marked S for Endo H sensitive), indicative of tyrosinase with high mannose forms characteristic of ER resident species (Halaban *et al*, 1997). These results showed that the processing of tyrosinase in the heterologous COS7 cellular system was not optimal.

In human melanoma cells (501 mel), a large portion of TYR-FLAG was also sensitive to Endo H digestion (**Fig 1*A***, *lanes 5–8*), consistent with the previously characterized folding defect for native tyrosinase in these cells (Halaban *et al*, 1997; 2001). The same membrane was blotted successively with a monoclonal antibody against FLAG (**Fig 1*A***, *lanes 5–6*, α-FLAG) and then against tyrosinase (**Fig 1*A***, *lanes 7–8*, α-TYR). This showed that the chimeric protein behaved similarly to the endogenous wild-type human protein, and that the FLAG epitope encoding for 19

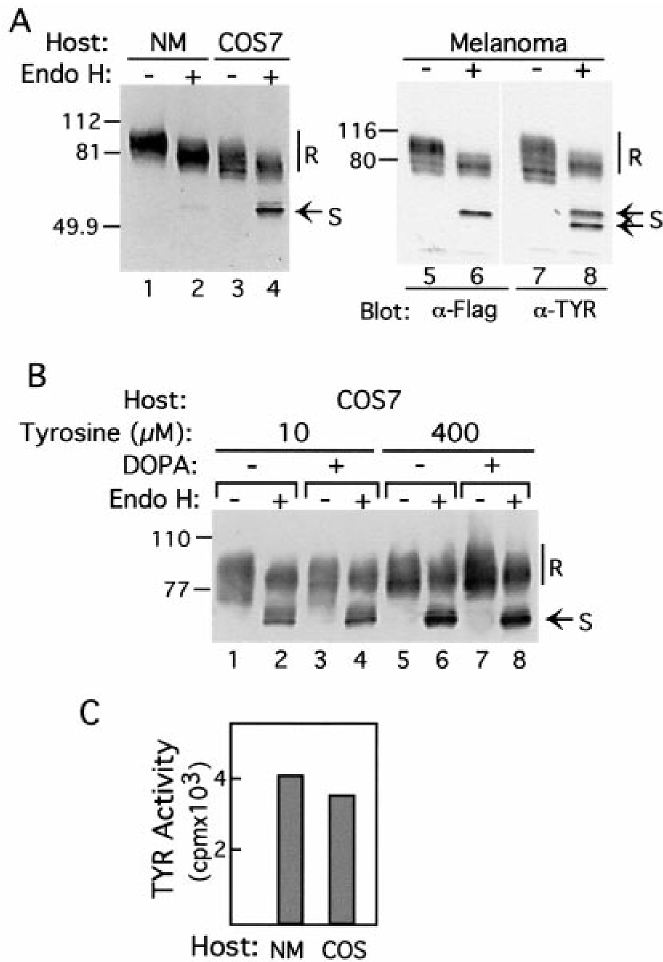

Figure 1. TYR-FLAG displays characteristics of native TYR protein. (*A*) and (*B*) Western blots with anti-FLAG (*A*, *lanes 1–6*; *B*) or anti-TYR monoclonal antibody (T311) showing nondigested (–) or Endo H digested (+) TYR-FLAG expressed in wild-type mouse melanocytes (NM), COS7, or human melanoma (501 mel). Endo H digestions were performed with precipitated material using WGA bound beads (*A*, *lanes 1–4*, and *B*) or antityrosinase (goat M-19, *A*, *lanes 5–8*). At the end of Endo H treatment, proteins were separated in 8% (*A*, *lanes 1–4*, and *B*) or 6% (*A*, *lanes 5–8*) polyacrylamide gels. The *bar* and *arrows* indicate Endo H resistant (R) and sensitive (S) tyrosinase proteins, respectively. Molecular weight markers are designated in kDa. (*C*) Tyrosinase activity in TYR-FLAG expressing cells. Assays were performed with anti-FLAG immunoprecipitated material from mouse melanocytes (NM), or COS7 whole cell lysates. Data are expressed as mean duplicates of total cpm minus blanks (reactions devoid of any cell extract). Tyrosinase activity in parallel assays using nontransfected cells was as in blanks.

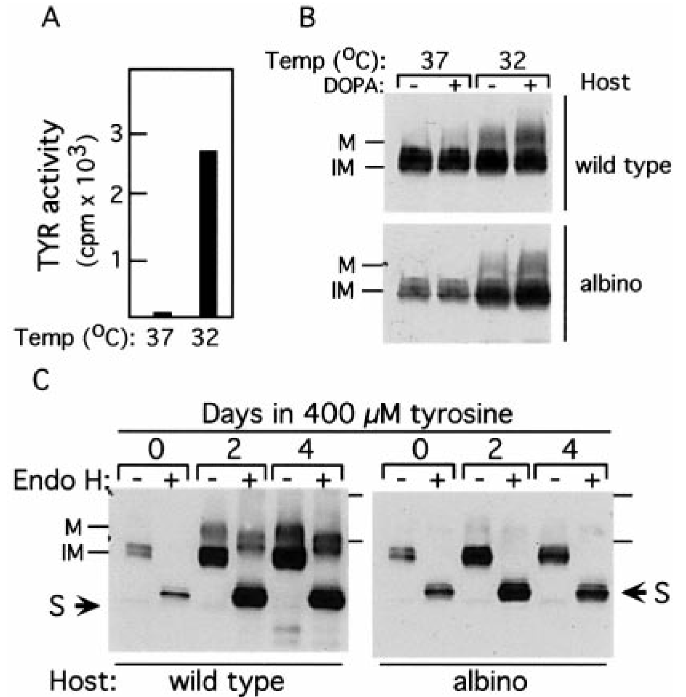

Figure 2. DOPA/tyrosine enhanced the stability and maturation of TYR-R402Q albino mutant. (*A*) TYR-R402Q-FLAG enzymatic activity expressed in wild-type mouse melanocytes. Transfected melanocytes were kept at 37°C, or shifted to 32°C for 24 h (as indicated). Chimeric proteins were precipitated from cell extracts (250 μg protein per assay) with an anti-FLAG monoclonal antibody and bead-bound materials were subjected to tyrosinase assay. Data are means of duplicate assays expressed as total cpm. Standard deviation did not exceed 10% of total counts. (*B*) Maturation of TYR-R402Q-FLAG expressed in wild-type and albino melanocytes in response to downward temperature shift and DOPA. Melanocytes were exposed to the experimental conditions for 24 h, and equal amounts of protein (40 μg per lane) were subjected to Western blotting with anti-FLAG monoclonal antibody. *Bars* indicate mature (M) and immature (IM) TYR proteins. (*C*) Stabilization and maturation of TYR-R402Q-FLAG in response to tyrosine. Melanocytes (wild-type and albino) expressing TYR-R402Q-FLAG were grown continuously in regular Ham's F-10 medium containing 10 μM tyrosine (0), or in Ham's F-10 medium supplemented with 400 μM tyrosine for 2 or 4 d (as indicated). Equal amounts of cell extracts (500 μg per assay) were subjected to bead-bound WGA precipitation, followed by digestion without (–) or with (+) Endo H. TYR-R402Q-FLAG was detected by Western blotting with anti-FLAG monoclonal antibody. *Bars* indicate mature and immature TYR species, and *small arrow* marks deglycosylated, Endo H digested TYR-R402Q-FLAG (S). *Bars* on the right-hand side indicate 110 kDa and 77 kDa marker proteins.

amino acids caused an upward electrophoretic mobility shift of the expected 2 kDa (**Fig 1*A***, *lanes 6*, *8*, marked S).

The accumulation of TYR-FLAG in COS7 cells after incubation in high tyrosine and DOPA (**Fig 1*B***) indicated that the chimeric protein responded to its substrates and cofactor in a fashion similar to that of native human TYR in melanoma cells (Halaban *et al*, 2001). Finally, TYR-FLAG possessed normal function as evidenced by the presence of tyrosinase activity in COS7 whole cell lysates and in anti-FLAG immunoprecipitated material from transfected mouse melanocytes (**Fig 1*C***), and by the accumulation of melanin in transfected COS7 cells (not shown). Taken together, these results confirmed that the C-terminal FLAG tag on human TYR did not impair TYR processing and activity when expressed in the three different cell types.

DOPA/tyrosine-induced maturation of the natural variant TYR-R402Q We explored the effect of DOPA/tyrosine on the naturally existing temperature-sensitive TYR-R402Q variant that in combination with an albino mutant can cause albinism (Tripathi *et al*, 1991; Spritz, 1994; Halaban *et al*, 2000). The temperature sensitivity of TYR-R402Q-FLAG chimeras was validated by measuring tyrosinase activity in anti-FLAG immunoprecipitated proteins derived from transfected mouse melanocytes grown at the nonpermissive and permissive temperature (**Fig 2*A***).

Enhanced maturation of TYR-R402Q-FLAG was observed in response to DOPA (**Fig 2*B***), or increased tyrosine concentration (**Fig 2*C***). Complex carbohydrate modification was enhanced at the permissive temperature (32°C) in the presence of DOPA (**Fig 2*B***), and at the nonpermissive temperature (37°C) with a high tyrosine concentration (400 μM) when the mutant was expressed in wild-type but not in albino melanocytes (**Fig 2*C***). **Figure 2(*C*)** also

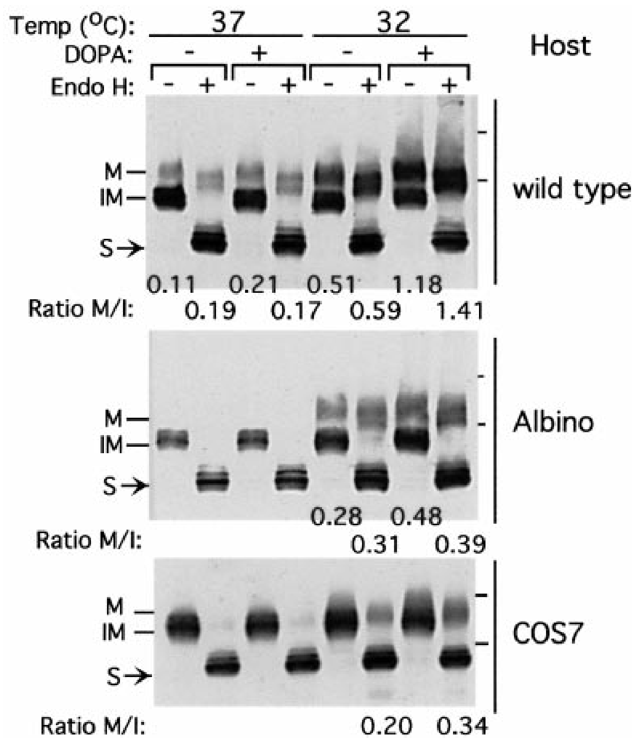

Figure 3. Maturation of TYR-R402Q-FLAG is promoted by endogenous tyrosinase activity and is not impeded by the albino mutant. Mouse melanocytes (wild-type and albino) and COS7 cells expressing TYR-R402Q-FLAG were incubated in medium supplemented with 200 μM tyrosine, in the absence (–) and presence (+) of DOPA (50 μM) at the nonpermissive (37°C) and permissive (32°C) temperature overnight (16 h). Bead-bound WGA material precipitated from equal amounts of cell extracts (250 μg) was treated (+) or not treated (–) with Endo H. Reaction products were subjected to Western blotting with anti-FLAG monoclonal antibody. Numbers under lanes represent the band density ratio of mature to immature (M per I) glycoforms as assessed by NIH image analyzer.

shows that tyrosine induced the accumulation of the immature Endo-H-sensitive TYR-R402Q-FLAG regardless of the genetic background of the host melanocytes. This accumulation is probably due to increased stability and resistance to proteolytic degradation (Halaban *et al*, 1997).

The inefficient maturation of temperature-sensitive proteins in albino *versus* wild-type melanocytes could be caused by the interference in the folding or oligomerization generated by a dominant misfolded and ER retained host mutant TyrC85S (Halaban *et al*, 2000), rather than by the positive effect generated from wild-type tyrosinase activity. We therefore assessed the maturation of TYR-R402Q-FLAG expressed in COS7 cells, which lack endogenous tyrosinase, in response to DOPA and temperature shift. These results were compared with TYR-R402Q-FLAG expressed in wild-type and albino melanocytes. Western blot analyses with anti-FLAG monoclonal antibody showed that processing in the albino background was similar to that in COS7 cells (**Fig 3**). The ratio of mature to immature R402Q-TYR-FLAG in these two cell lines, as indicated by the densities of the respective bands assessed with an NIH image analyzer, was similar and was smaller than FLAG-tagged R402Q-TYR expressed in wild-type mouse melanocyte cells (**Fig 3**, *M/I* ratios below bands). These results indicate that the presence of the tyrosinase cofactor produced by the enzymatic activity of the host tyrosinase may be the driving force behind the proper maturation.

Elimination of specific N-linked glycosylation sites hinders processing of tyrosinase Glycans can be important for proper folding of glycoproteins and elimination of a particular site can confer temperature sensitivity for folding (Hebert *et al*, 1997; reviewed in Helenius and Aebi, 2001). To assess the contribution of each of the seven glycosylation sites to TYR conformation, glycan-deficient mutants were created. The Thr or Ser in each of the human TYR N-glycosylation consensus sites was changed to Ala, except for Thr 373, which was substituted with Lys to mimic the common T373K natural OCA1 mutation as described previously (Újvári *et al*, 2001). The wild-type and mutant cDNAs were then ectopically expressed as FLAG-tagged proteins in wild-type or albino mouse melanocytes.

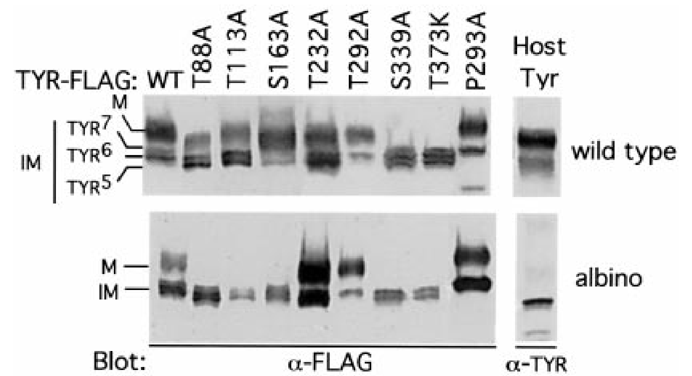

Figure 4. Elimination of specific glycans hinders processing to different degrees in a cell-type-dependent manner. TYR-FLAG (wild-type and substitution mutants at positions as indicated) were expressed in wild-type and C85S albino mouse melanocytes. Anti-FLAG Western blots were performed on WGA bound material (α-FLAG). Host tyrosinase proteins are represented by Western blot of whole cell lysates with antityrosinase (α-TYR, goat M-19). Tyrosinase proteins with five, six, and seven glycans are indicated on the left-hand side. M and IM mark mature and immature protein bands, respectively.

Western blot analysis showed that elimination of each glycan individually produced proteins that migrated by SDS-PAGE faster than their wild-type counterpart (**Fig 4**). The immature ER form displayed a 3 kDa size reduction consistent with the loss of the expected mass of a single high mannose glycan (**Fig 4**, *top panel*, bands marked IM for immature). Because six or seven glycans are processed on TYR (Újvári *et al*, 2001), the resulting mutant proteins displayed a doublet consisting of five or six glycans, except for the T292A mutant. This mutant consistently produced a protein with six glycans due to elimination of the variable site whose glycosylation is suppressed ≈50% of the time by an adjacent proline (Újvári *et al*, 2001). Likewise, elimination of this adjacent proline, Pro293, produced a protein that maintained seven glycans (**Fig 4**, P293A). Complete digestion of the doublet protein from several mutants by Endo H confirmed that it is the ER immature form (data not shown).

When expressed in wild-type mouse melanocytes, the seven glycosylation mutants could be divided into three groups with respect to their ability to mature into fully processed ≈90 kDa protein that had received complex sugars in the Golgi (**Fig 4**, *top panel*): (i) normally processed (S163A, T292A, and P293A); (ii) partially processed (T88A, T113A, and T232A); and (iii) unprocessed (S339A and T373K). When expressed in albino melanocytes, however, the deficiency in maturation was more extensive (**Fig 4**, *bottom*). Wild-type TYR-FLAG and the chimeric proteins carrying the T232A, T292A, and P293A substitutions remained the most efficiently processed proteins, although in some cases a larger portion persisted as the ER unprocessed form compared to proteins expressed in wild-type melanocytes (**Fig 4**, *bottom panel*, bands marked IM). The category of unprocessed proteins extended to include T88A, T113A, and S163A TYR-FLAG chimeric proteins. Only T232A, T292A, and P293A matured to the same extent in the albino background as in wild-

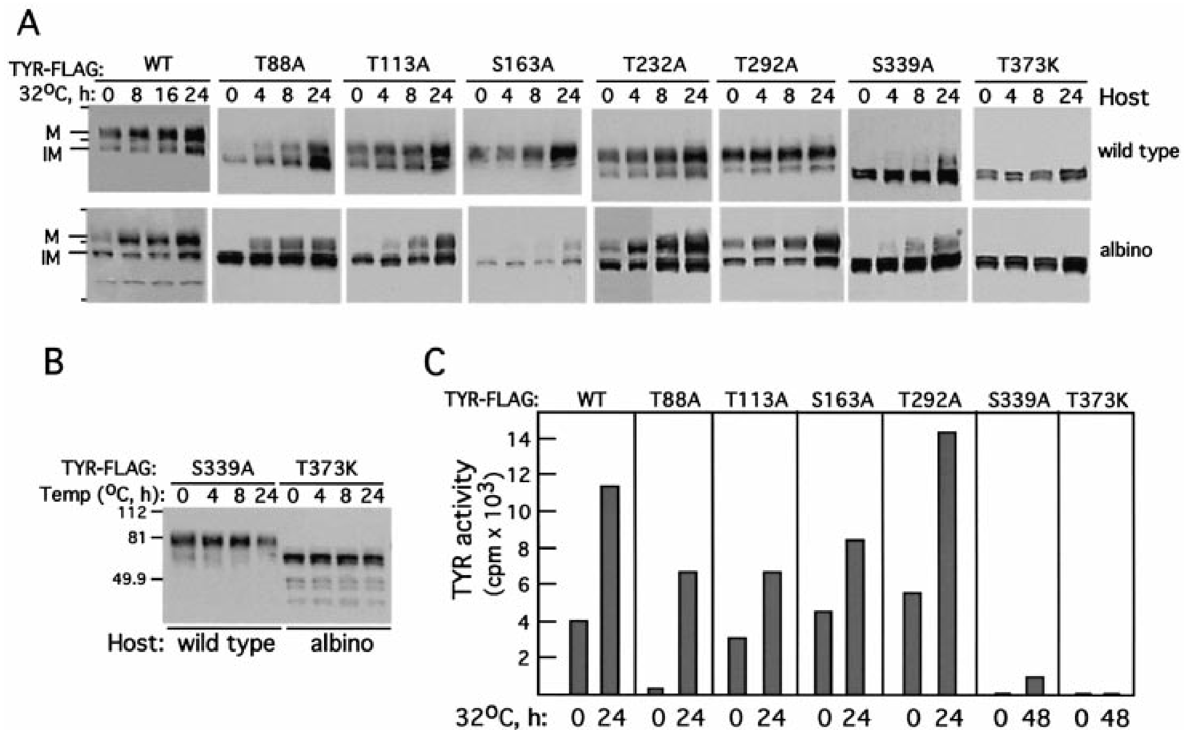

Figure 5. Stabilization of TYR-FLAG by downward temperature shift. Mouse melanocytes (wild-type and albino C85S mutant) expressing chimeric FLAG-tagged TYR proteins (wild-type or glycan deficient, as indicated) were grown continuously at 37°C (0) or shifted to 32°C for the indicated time interval. (*A*) Cell lysates from each transfectant were precipitated with WGA bound beads and subjected to Western blotting with anti-FLAG monoclonal antibody. Equal amounts of protein were used with each transfectant. Protein ranged from 400 to 700 μg per assay. (*B*) Mouse host proteins (wild-type and C85S mutant) in transfected cells. The membranes presented in (*A*) were successively blotted with goat antimouse tyrosinase antibodies (M-19). Shown are two representative Western blots of S339A and T373K transfected melanocytes. The respective mouse tyrosinase in the other blots appeared similar. (*C*) Tyrosinase activity in anti-FLAG immune precipitates. Cell extracts containing 200–250 μg protein (equal amount for each transfectant) were subjected to precipitation with FLAG M2 monoclonal antibody, and bead-bound material was assayed for tyrosinase for 30 min at 37°C. Data are averages of duplicate assays and are expressed as total cpm minus blanks. Variation between samples did not exceed 10% of total counts.

type melanocytes, suggesting that these mutations stabilized the protein. Host tyrosinase proteins (Tyr) are represented by Western blot of whole cell lysates with goat antityrosinase (α-TYR). The wild-type and C85S albino mutant mouse tyrosinase each displayed electrophoretic mobility characteristic of fully processed and Endo-H-sensitive proteins as described previously (Halaban *et al*, 2000; Toyofuku *et al*, 2001).

The various TYR-FLAG chimeric proteins also converted the amelanotic albino melanocytes to pigmented cells with different efficiencies. Although the level of pigmentation was dependent on the fraction of cells expressing the ectopic genes, cells expressing the T292A mutation were consistently darker than those expressing the wild-type form.

Stabilization of glycosylation mutants at 32°C The observations that the instability of glycan-deficient mutants was more prevalent when expressed in albino compared to wild-type host melanocytes suggested that proper maturation of TYR can be enhanced by the presence of wild-type TYR for a portion of the mutants. We therefore examined maturation in response to downward temperature shift from 37°C to 32°C. Time course analysis showed that the total level of most of the chimeric proteins increased within 4 h at 32°C when expressed in the wild-type mouse melanocytes. This effect was most apparent for wild-type, T88A, T113A TYR-FLAG chimeras (**Fig 5*A***, *top panel*). In contrast, there was no change in the level of FLAG-tagged TYR-T292A, indicating again that elimination of this glycan produced a protein with optimal stability. The reduction in temperature resulted in a slight appearance in Golgi processing of S339A after 24 h that was not seen at the higher temperature, whereas no band shift was observed for TYR-T373K-FLAG (**Fig 5*A***, *top panel*, S339A and T373K). A similar hierarchy of enhanced TYR processing after downward temperature shift was observed when the proteins were ectopically expressed in albino mutant melanocytes (**Fig 5*A***, *bottom panels*). The largest effect was seen with T88A, T113A, and S163A, which changed from being completely unprocessed at 37°C in albino cells, to being partially processed at 32°C.

Successive immunoblotting of the same membranes (**Fig 5*A***) with goat polyclonal antibodies directed against mouse tyrosinase (M-19) revealed that ectopic expression of human FLAG-TYR albino mutant proteins did not affect the migration pattern or stability of endogenous wild-type or C85S albino mutant mouse tyrosinase (**Fig 5*B***). In addition, as expected, the downward temperature shift did not alter the characteristic levels of glycan processing of each host protein. Wild-type and C85S albino mouse tyrosinase appeared as fully processed 80 kDa and immature 70 kDa glycoforms, respectively, as reported before (Halaban *et al*, 2000).

The increase in protein abundance and maturation was accompanied by a similar increase in tyrosinase activity and correlated with the intrinsic level in each construct (**Fig 5*C***). The highest TYR activity was detected in TYR-T292A, whereas none was detected in TYR-T373K FLAG chimeras regardless of temperature, confirming previous results (Park *et al*, 1993). FLAG-tagged

TYR-T88A displayed a dramatic increase in activity after 24 h downward temperature shift, in agreement with induced processing observed in immunoblots. Likewise, residual enzymatic activity was measured in TYR-S339A after 2 d incubation at low temperature, paralleling the low levels of maturation under similar conditions. We therefore concluded that elimination of specific glycans induced varying degrees of temperature sensitivity for maturation, a defect that was eased in the presence of wild-type protein.

DOPA/tyrosine-induced maturation of temperature-sensitive glycan-deficient tyrosinase Because TYR-FLAG proteins were destabilized particularly when expressed in albino melanocytes that are null for TYR but normal for all other melanocyte-specific proteins, we attempted to restore proper maturation with tyrosine and DOPA. Toward this goal, the transfected melanocytes were incubated with 400 μM tyrosine for 2 d, or with DOPA (50 μM) for 16 h. Western blot analysis of chimeric proteins expressed in wild-type mouse melanocytes showed that tyrosine and DOPA induced maturation and accumulation of TYR-FLAG chimeric proteins in a fashion similar to downward temperature shift (**Fig 6*A*, *B***, *top panel*). As before, the glycan-deficient T88A and T113 TYR-FLAG were most responsive, whereas the S339A and TYR-T373K mutants were slightly or unaffected, respectively remaining largely unprocessed regardless of the treatment. Tyrosine and DOPA also stabilized wild-type TYR-FLAG, reminiscent of the response of native tyrosinase in melanoma cells (Halaban *et al*, 2001), whereas T292A TYR-FLAG remained unchanged, consistent with it having already reached its optimal maturation level.

The role of tyrosinase activity in this process was further confirmed by incubating transfected albino melanocytes with DOPA. In this genetic background, DOPA stabilized chimeric proteins that possess residual levels of active mature protein at the time of the assay (**Fig 6*B***, *bottom panel*, compared to **Figs 4, 5**). For example, maturation of T88A TYR-FLAG present as the 70 kDa unprocessed glycoform with the lowest level of tyrosinase activity (**Fig 5**) was not rescued by DOPA when expressed in the albino background, whereas processing of the T113A and S163A with low levels of mature glycoforms and endogenous activity was enhanced. In contrast, the mutants S339A and T373K did not respond to DOPA stimulation and remained unprocessed regardless of genetic background. Altogether, the results showed that folding of temperature-sensitive glycan-deficient mutants can be enhanced by the accumulation of tyrosinase reaction products at certain threshold levels generated from self-activation or from coexpressed wild-type protein.

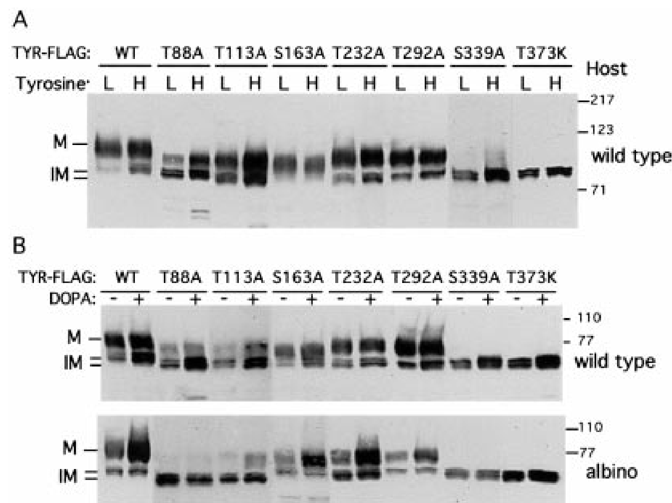

Figure 6. Maturation of TYR-FLAG wild-type and mutant proteins in response to tyrosine or DOPA. Western blotting with anti-FLAG monoclonal antibody of proteins precipitated from cell extracts with bead-bound WGA. (*A*) Mouse melanocytes (wild-type) expressing wild-type or glycan-deficient TYR-FLAG chimeric proteins (as indicated at the top) were grown continuously in Ham's F-10 medium containing 10 μM tyrosine. Parallel cultures were either kept in Ham's F-10 medium (L, for low tyrosine concentration), or were subjected to Ham's F-10 medium supplemented with 400 μM tyrosine (H, for high tyrosine concentration). Cultures were harvested 2 d later. (*B*) Parallel cultures of mouse melanocytes (wild-type or albino, as indicated), expressing wild-type or glycan-deficient TYR-FLAG chimeric proteins, were incubated without (–) or with (+) DOPA (50 μM) for 1 d. Protein size markers are indicated in kDa.

DISCUSSION

We have previously shown that transport of wild-type tyrosinase through the vesicular network, in particular from the ER to the Golgi complex, is facilitated by its own cofactor and substrate (Halaban *et al*, 2001). Agents that induced tyrosinase activity and the subsequent production of DOPA also enhanced its trafficking from the ER to the Golgi, and possibly beyond to the melanosomes. These observations are now extended to include temperature-sensitive mutants of tyrosinase. The release of these mutant proteins from the ER to the Golgi complex was dependent on their intrinsic residual enzymatic activity and was further enhanced by the activity of wild-type tyrosinase in the host melanocyte. Coexpression of mutant proteins with wild-type tyrosinase alleviated their conformational defect and enhanced transit from the ER to the Golgi by an active process that was dependent on the level of endogenous and ectopic tyrosinase activity manipulated by tyrosine and DOPA. These observations not only are significant for understanding the myriad of levels of pigmentation displayed by individuals carrying the autosomal recessive OCA1B or OCA2 mutations, but also may provide a mechanism to enhance pigmentation in individuals with residual TYR activity.

The Golgi processing of the natural temperature sensitive variant TYR-R402Q even at the nonpermissive temperature when coexpressed as FLAG-tagged protein with wild-type tyrosinase may explain the known phenotype of carriers of this mutation. The R402Q substitution is found at high frequency in the normal Caucasian population. Individuals that carry this allele in combination with null TYR, however, have reduced pigmentation during

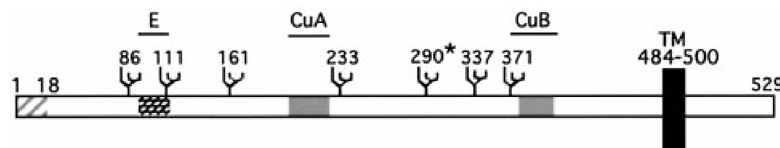

Figure 7. Schematic representation of human TYR. The empty horizontal rectangle represents TYR 529 amino acid (aa) mature peptide (accession number XM_027058). The hatched N-terminus box indicates the 18 amino acid signal peptide, the crossed area the epidermal-growth-factor-like domain (E), the gray areas the Cu binding sites A and B, and the dark vertical bar the hydrophobic core transmembrane domain (TM). The positions of the seven N-linked oligosaccharides are represented by the branched structures above the bar, with numbers indicating the position of Asn. The inefficient N-linked glycosylation at position 290 is denoted with an asterisk.

embryogenesis, are born with ocular albinism, but develop pigment later on in life (Oetting and King, 1999). The normal pigmentation in homozygous R402Q carriers and the differences in melanin synthesis before and after birth in heterozygotes with null mutations could be explained by gradual accumulation of DOPA with time, inducing stabilization and upregulating the levels of the protein after birth.

Our analysis of glycan-deficient mutants defined the role of each of the seven N-linked glycosylation sites in tyrosinase maturation (**Fig 7**). Two sites at position 337 and 371 were proven to be indispensable for proper processing when eliminated. The Asn371-X-T (the only N-linked glycosylation site known to be eliminated by natural mutations) is the site of several substitutions identified in individuals with OCA1, which include the N371Y, N371T, and T373K exchanges (reviewed in Oetting, 2000). The mutant N371T and T373K proteins cause a severe OCA1 albino phenotype (reviewed in Oetting, 2000). On the other hand, TYR N371Y causes an OCA2 type of albinism, i.e., allowing some formation of yellow pigment (Passmore *et al*, 1999). Analysis of an additional mutation at this site, N371Q, produced by site directed mutagenesis, also showed that the severity of misfolding is modified by the type of amino acid substitution, because the Asn to Gln exchange allowed low levels of maturation (Xu *et al*, 2001). The 337 and 371 N-linked glycosylation sites in TYR flank the copper binding domain B (**Fig 7**), and a cluster of missense mutations in this region are the cause of OCA1. It is possible that the two glycans are needed to preserve the secondary structure that maintains the two copper atoms in binding sites A and B at the right distance to form a peroxide complex with molecular oxygen (Gerdemann *et al*, 2002). Elimination of N-terminal glycans in Tyrp1, a protein with high homology to TYR, at positions 304 or 385 also produced a protein that was retained in the ER (Xu *et al*, 2001). This suggests, by analogy to TYR, that these sites define a region in Tyrp1 critical for enzymatic activity, though the precise nature of Tyrp1's activity is still disputed.

Elimination of glycans at positions 230 or 290 in TYR did not reduce the stability of the protein below that of wild-type protein. Moreover, elimination of the 290 site with the T292A alteration was not only dispensable for folding but also conferred a more stable conformation on tyrosinase. Interestingly, this N-linked glycosylation consensus site is not present in mouse tyrosinase (Kwon *et al*, 1988; Muller *et al*, 1988; Yamamoto *et al*, 1989), a protein that is more efficiently processed than its human homolog (Halaban *et al*, 2000). Furthermore, the proline at position 293 suppresses carbohydrate transfer to the N290 site producing the doublet (TYR6/TYR7) observed for the ER glycoforms (Újvári *et al*, 2001, and **Fig 4**). The TYR6 form is more prevalent in the rapidly translating melanoma cells, however, for which TYR maturation is compromised due to the presence of a hostile maturation environment (Újvári *et al*, 2001; Halaban *et al*, 2002).

The remaining N-terminal N-linked glycosylation sites, N86, N111, and N161, embedded in or next to the epidermal-growth-factor-like motif of TYR (Oetting, 2000), produced temperature-sensitive mutants for folding, particularly apparent when expressed in the albino background. Whereas the FLAG-tagged proteins TYR-T88A, TYR-T113A, and TYR-S163A remained as ER-misfolded glycoforms when expressed in albino melanocytes grown at 37°C, they were transported to the Golgi at this nonpermissive temperature when expressed in wild-type melanocytes. Interestingly, of the three, TYR-T88A-FLAG displayed the most severe conformational defect. The T88A is adjacent to a critical cysteine C89 and is located in a hot spot of OCA1 mutations, suggesting again a critical region for TYR folding.

One should note that these findings are in direct conflict with results employing mouse tyrosinase, which possesses 85% sequence identity with the human protein. Branza-Nichita *et al* (2000) reported that the glycosylation sites at 111 and 161 in mouse tyrosinase are not occupied, and that deletion of the 371 site (which is associated with severe OCA1) results in the production of tyrosinase with 63% of its relative enzymatic activity.

If tyrosinase is a homo-oligomer, the host cellular wild-type tyrosinase may assist in the maturation of the transfected and tagged proteins by pushing the oligomerization reaction towards product formation regardless of its intrinsic catalytic activity. Alternatively, the export of the temperature-sensitive mutants could be passively enhanced by binding to the nascent wild-type tyrosinase in the ER without obtaining the active conformation. If that were the case, the transport to the Golgi should have been occurring regardless of temperature, the levels of tyrosine, the presence of DOPA, or the intrinsic residual enzymatic activity of the mutant proteins, because wild-type mouse tyrosinase is continuously fully processed in these cells.

Altogether, our results demonstrate that tyrosinase reaction products enhance proper conformation not only of wild-type but also of mutant tyrosinase proteins that possess residual enzymatic activity. They imply that tyrosinase is destabilized when its own reaction products are depleted. This concept is supported by our previous observations that downregulation of tyrosinase activity in tyrosinase-positive amelanotic melanoma cells is the cause for loss of pigmentation.

Electronic database information:

1 ADB, The Albinism Database: URL http://www.cbc.umn.e-du:tad.

2 OMIM, Online Mendelian Inheritance in Man, Center for Medical Genetics, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), http://www.ncbi.nlm.nih.gov/omim/

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