A Common Temperature-sensitive Allelic Form of Human Tyrosinase Is Retained in the Endoplasmic Reticulum at the Nonpermissive Temperature*

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Oculocutaneous albinism type 1TS is caused by mutations that render the melanocyte-specific enzyme tyrosinase temperature-sensitive (ts); the enzyme is inactive in cells grown at 37 °C but displays full activity in cells grown at 31 °C. To distinguish whether the ts phenotype of the common R402Q variant of human tyrosinase is due to altered enzymatic activity or to misfolding and a defect in intracellular trafficking, we analyzed its localization and processing in transiently transfected HeLa cells. R402Q tyrosinase accumulates in the endoplasmic reticulum (ER) at 37 °C but exits the ER and accumulates in endosomal structures in cells grown at 31 °C. The inability of the R402Q variant to exit the ER is confirmed by the failure to acquire endoglycosidase H resistance at 37 °C and cannot be accounted for solely by enhanced proteasome-mediated degradation. ER retention at 37 °C is mediated by the luminal domain of R402Q tyrosinase, is not dependent on tethering to the membrane, and is irreversible. Finally, a wild-type allelic form of tyrosinase is partially ts in transiently transfected HeLa cells. The data show that human tyrosinase expressed in non-melanogenic cells folds and exits the ER inefficiently and that R402Q tyrosinase exaggerates this defect, resulting in a failure to exit the ER at physiologic temperatures.

Oculocutaneous albinism type 1 (OCA1)† comprises a group of human disorders characterized by reduced or absent skin pigmentation, vision defects due to reduced optical pigmentation and to misrouting of optic nerve fibers, and enhanced sensitivity to skin and optical cancers (1). These disorders are caused by germline mutations within the coding region of the melanocyte-specific gene product tyrosinase (2–4). Tyrosinase, a key enzyme in the synthesis of melanin (reviewed in Ref. 5) is a type I integral membrane glycoprotein that is specifically expressed in cells of the melanocyte lineage, where it localizes to a specialized late endosome-like compartment, the melanosome (6). Within melanosomes, tyrosinase initiates melanin formation by enzymatic tyrosine hydroxylase and L-3,4-dihydroxyphenylalanine (DOPA) oxidase activities that are associated with its lumenal domain; a 5,6-dihydroxyindole oxidase activity, also a property of the tyrosinase lumenal domain, may contribute to a subsequent step in melanin synthesis (reviewed in Ref. 1). Lack of tyrosinase activity results in the absence of melanin formation due to the inability to form the melanin precursors, L-3,4-dihydroxyphenylalanine and L-3,4-dihydroxyphenylalanine quinone. OCA1 is a direct result of the absence or reduction of melanin.

Different germline mutations within the tyrosinase coding region underlie OCA1 disorders of varying severity. In one unusual subset of OCA1, OCA1 TS, the mutations render tyrosinase temperature-sensitive (ts). Consequently, melanin synthesis only occurs in cooler areas of the body, such as the arms and legs. The resultant pattern of peripheral pigmentation is analogous to that of the Siamese cat and the Himalayan mouse (7, 8). A number of ts variants of tyrosinase have been molecularly cloned. One variant results from a single base missense mutation that alters an arginine at amino acid position 402 to a glutamine (R402Q). The allele encoding the R402Q variant of tyrosinase is extremely prevalent, representing about 15% of the gene pool among Caucasians (8). Studies in cultured transfected cells of the R402Q variant and the similar but less prevalent R422Q variant have shown that they have ts activity; transfected cells grown at 37 °C display none of the described tyrosinase enzymatic activities, whereas transfected cells grown at 31 °C display all three activities, comparable with that of wild-type (9, 10). Both variants contain mutations that affect residues close to a defined copper binding site within the lumenal domain (11). Nevertheless, the molecular mechanisms by which these mutations render the variants ts have not been elucidated.

ts enzyme activity is a reflection of a conformational defect at elevated temperatures and stabilization of an active conformation at reduced temperatures. Defects at the restrictive temperature may be subtle such that only specific molecular contacts required for function are lacking, as in the ts mutants of dynamin from Drosophila melanogaster (12) and humans (13). Alternatively, defects may be dramatic such that the ts protein is grossly misfolded at the restrictive temperature (14), as seen in a number of genetic diseases (15–18). Such ts mutants would
be expected to be substrates for the cellular quality control system. The quality control system recognizes misfolded or unassembled polypeptides and either sequesters them or targets them for degradation to prevent large scale protein aggregation and consequent loss of cellular function (19, 20).

The major site for quality control within the secretory pathway is the endoplasmic reticulum (ER) (21). Within the ER, newly synthesized polypeptides are retained by the activity of resident chaperone proteins until they are fully folded and assembled, at which point they are released for transport to distal secretory compartments (19, 22, 23). Defective polypeptides that fail to fold and/or assemble are retained within the ER and/or degraded by ER-associated or cytosolic proteolytic systems, particularly the proteasome, after reverse translocation into the cytosol (24). Should partially folded or assembled proteins escape ER quality control and progress to distal secretory compartments, they can be recognized at one of several secondary sites for quality control, as evidenced by several examples in yeast (25, 26), and targeted to lysosomes/vacuoles for degradation. Finally, specific chaperones may act to prevent unfolding and degradation within late endosomal/lysosomal compartments (27, 28). Thus, quality control can be exerted at multiple sites within the secretory/endosomal system. Enzymes with gross defects in folding would be expected to be subject to one or all of these quality control checkpoints.

For ts gene products that result in human disorders, distinguishing between subtle and dramatic protein folding defects and determining the intracellular site at which the ts phenotype emerges is important in designing therapeutics to address clinical symptoms. Here, we analyze the localization and processing of the R402Q variant of human tyrosinase and show that the ts phenotype is due to a defect in protein folding that prevents ER exit. The partial ts phenotype of a wild-type (WT) allelic form of tyrosinase and the lack of an apparent significant increase in ER-associated degradation of the R402Q variant suggest that the R402Q variation exaggerates an inefficient folding process inherent in human tyrosinase when expressed in non-melanogenic cells.

**EXPERIMENTAL PROCEDURES**

**Cells, Culture Conditions, and Transfections—**HeLa cells were maintained as described previously (29). Cells were transiently transfected using calcium phosphate precipitation as described (30) using 3–5 μg of total DNA into a 6-well dish. After transfection, cells were incubated overnight at 37 °C, the medium was changed into pCMV8.1 (31). TTT-HA and HA-tagged chimeras between Tac and tyrosinase were constructed by overlapping polynucleotide chain reaction (32) using pCI-tyrR402Q and Tac-Lamp1 (33) as templates. For chimeric proteins with the tyrosinase lumenal domain, an EcoRI-BgII fragment containing most of the coding region for the lumenal domain of R402Q tyrosinase was subcloned into pCMV8.1 to create pY_YWxx. Polynucleotide chain reaction-generated BgII-XhoI inserts were subcloned into pYWxx or Tac-Lamp1 to generate the chimeric expression vectors. Details of the polynucleotide chain reaction will be provided upon request.

In all chimeric constructs, the hydrophilic amino acids surrounding the endogenous transmembrane domain were left intact. Y_iwY-THA and Y_iwTT-HA were generated by excising the EcoRI-BgII fragment of Y_iwY-THA and Y_iwTT-HA, respectively, and replacing it with the EcoRI- BgII fragment of pCMV8.1-tyr501-1. Construction of TTY is described elsewhere (39). Constructs were verified by automated DNA sequencing at the Nucleic Acid Core Facility of Children’s Hospital of Philadelphia. TT-lamp1 has been previously described (30).

**Immunofluorescence Microscopy—**Cells grown on coverslips were fixed with 2% formaldehyde in PBS and stained with unlabeled primary antibodies and fluorescein isothiocyanate- and lissamine-rhodamine-conjugated secondary antibodies as described (29). Cells were analyzed on a Zeiss Axiosplan microscope, photographed using Kodak ASA 400 film, and digitized using a Nikon LS-1000 slide scanner. Images were processed using Adobe Photoshop software. Images in Fig. 1 are from cells transfected with 3 μg of WT or R402Q tyrosinase/well in a 6-well dish. Where indicated, cells were treated with 50 μg/ml cycloheximide (CHX) for 2 h before fixation.

**Metabolic Labeling and Immunoprecipitation—**HeLa cells transfected in 10-cm dishes were harvested, pelleted, and labeled for 30 min with [35S]methionine/cysteine labeling mix (NEN Life Science Products) and chased with excess methionine and cysteine essentially as described (30), except that all procedures were done at the indicated temperatures. Where indicated, 50 μM ammonium chloride or 20 μM methionine methyl ester (MME) was added to the chase medium. Cells were washed once with ice-cold PBS, and cell pellets were stored at −70 °C. Immunoprecipitations were performed essentially as described (29). Briefly, cells were resuspended in lysis buffer containing 1% Triton X-100 and a panel of protease inhibitors on ice for 30 min, then cleared of nuclei and cell debris by centrifugation. Lysates were pre-cleared by incubation with protein A-Sepharose beads (Amersham Pharmacia Biotech) and then sequentially immunoprecipitated using protein A-Sepharose beads that had been preadsorbed with 1 μl of normal rabbit serum followed by beads preadsorbed with 1 μl of pEP7 anti-tyrosinase serum. Beads were washed extensively with buffer containing 0.05% Triton X-100, once with PBS, and then divided into 3 aliquots that were either protein N-glycanase F (endoF, New England Biolabs, Beverly, MA), endoglycosidase H (endoH, New England Biolabs), or mock-endoH-treated according to the manufacturer’s instructions. Proteins were eluted from the beads with SDS sample buffer. Samples were fractionated by SDS-PAGE using gels containing 10% acrylamide. Dried gels were subjected to qualitative image analysis using a Molecular Dynamics Storm PhosphorImager and ImageQuant version 1.11 software.

**Western Blotting—**For experiments analyzing the effects of proteasome inhibitors, transfected HeLa cells were released from 10-cm plates by incubation in PBS, 10 mM EDTA, or trypsin, then resuspended in fresh culture medium lacking or containing 0.2% dimethyl sulfoxide with or without 50 μM MG-132 (Calbiochem) or 50 μM lactacystin (Calbiochem). Cells were incubated as indicated and then harvested by centrifugation, resuspended in PBS, divided into aliquots for endoglycosidase treatment, and then boiled with 0.5% SDS, 1% β-mercaptoethanol and heating to 95 °C for 10 min. The lysates were treated with DNase I at 37 °C for 15 min, reheat to 95 °C for 10 min, and then either enzymatically deglycosylated as above or mock-treated.

Expression and secretion of the polystisidine-tagged soluble tyrosinase constructs were analyzed as follows. HeLa cells were transfected with 15 μg of the desired construct/10-cm dish and incubated at 37 °C
isothiocyanate-conjugated anti-mouse Ig (e) and lissamine-rhodamine-conjugated anti-rabbit Ig. In e and f, cells were costained with AF8 (a monoclonal antibody to calnexin) and fluorescein isothiocyanate-conjugated anti-mouse Ig (f).

FIG. 1. Steady state localization of WT and R402Q tyrosinase at permissive and nonpermissive temperatures. HeLa cells transiently transfected with plasmids encoding WT (a and b) or R402Q tyrosinase (c–f) were grown on coverslips at 37 °C (a, c, e, and f) or 31 °C (b and d) and then fixed and processed for indirect intracellular IFM. Cells were stained with αPep7h antityrosinase serum (a–e) and lissamine-rhodamine-conjugated anti-rabbit Ig. In e and f, cells were costained with AF8 (a monoclonal antibody to calnexin) and fluorescein isothiocyanate-conjugated anti-mouse Ig (f).

overnight. The medium was then replaced, and the cells were incubated at the indicated temperature for an additional 24 h. Where indicated, 2 days post-transfection culture medium was replaced with medium containing 10 μg/ml CHX. Cells were incubated for 30 min at the initial temperature and then were either harvested or shifted to the final temperature and grown for 7 h in fresh medium supplemented with CHX, at which point both cells and culture medium were harvested. Culture medium was clarified by centrifugation and incubated with nickel nitrilotriacetic acid-Superflow beads (Qiagen, Chatsworth, CA) at 4 °C for 3 h at 4 °C. The suspension was divided into three aliquots, the beads were pelleted and washed once with PBS, and then either endoF-, endoH-, or mock-endoH-treated as above. Proteins were eluted from the beads with SDS sample buffer containing 100 mM EDTA. Cell lysates were prepared as described above following detachment of cells with PBS, 10 mM EDTA.

Samples were fractionated by SDS-PAGE using gels containing 10% acrylamide, and proteins were electrophoretically transferred to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA). The membranes were blocked in Blotto (PBS, 5% milk, 0.1% Tween 20), incubated with NY8K or αPep7h anti-tyrosinase serum diluted in Blotto, and then washed with PBS, 0.2% Tween 20. In some experiments, the membranes were then developed using a horseradish peroxidase detection system, in which membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin in Blotto and washed as above. Bound antibody was detected by ECL (Amersham Pharmacia Biotech), exposure to Reflection Film (NEN Life Science Products), and film processing using a Kodak M35A X-omat processor. For quantitative detection, membranes were developed using an alkaline phosphatase detection system. Membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin in Blotto, washed as above, developed using ECF (Amersham Pharmacia Biotech), and detected and quantitated using a Molecular Dynamics Storm 860 PhosphorImager with ImageQuant version 1.11 software.

RESULTS

The R402Q Variant of Human Tyrosinase Accumulates in the ER at the Nonpermissive Temperature—We have shown that a WT allelic form of human tyrosinase localizes predominantly to late endosomes and lysosomes when expressed in non-melanocytic cells (39). To determine whether tyrosinase with the R402Q variant is distinctly localized, we analyzed transiently transfected HeLa cells expressing WT or R402Q tyrosinase by IFM. As shown in Fig. 1a, WT tyrosinase localizes primarily to punctate, vesicular structures throughout the cytoplasm of HeLa cells, with some perinuclear concentration. These structures contain lamp1 and lamp2 (not shown), confirming their identity as late endosomes and lysosomes. In addition, WT tyrosinase is detected in reticular structures and the nuclear envelope. In contrast, R402Q tyrosinase localizes exclusively to the nuclear envelope and reticular structures throughout the cytoplasm (Fig. 1c). This pattern coincides with that of the ER resident chaperone protein, calnexin (Fig. 1, e and f), suggesting that the R402Q variant accumulates in the ER. Similar observations were made in several transfected cell lines, including the human melanoma line, MelJuSo (data not shown), suggesting that this localization pattern is specific to the R402Q variant and not merely a characteristic of tyrosinase expression in HeLa cells.

The R402Q variant was previously shown to be ts, such that extracts from transfected cells grown at 37 °C had defective tyrosinase activity, whereas extracts from cells grown at 31 °C had normal activity (10). We therefore tested whether the pattern of WT or R402Q tyrosinase localization is changed in HeLa cells grown at 31 °C. As shown in Fig. 1b, decreased growth temperature results in a slight increase in the vesicular form of WT tyrosinase accompanied by a decrease in reticular staining; this is consistent with enhanced stability and/or ER exit of WT tyrosinase. The effect of decreased temperature was much more dramatic for R402Q tyrosinase, which localized predominantly to vesicular structures in cells grown at 31 °C (Fig. 1d). Parallel experiments indicate that these vesicular structures contain lamp1 but lack calnexin (data not shown).

These observations suggest that although the R402Q variant is retained within the ER at the nonpermissive temperature, a significant fraction of it exits the ER and localizes to late endosomes in cells grown at the permissive temperature.

Failure of the R402Q Variant Glycoprotein to Mature at the Nonpermissive Temperature—To confirm the IFM results and begin to address the molecular basis for ER retention, we analyzed the behavior of WT and R402Q tyrosinase molecules in transfected cells using metabolic pulse-chase, immunoprecipitation, and SDS-PAGE. Transport of tyrosinase through the secretory pathway was monitored by following the maturation state of N-linked glycan chains using endoH and endoF, which cleave only high mannose or all-N-linked glycans, respectively. Because processing of N-linked glycans results in heterogeneity of tyrosinase migration by SDS-PAGE, acquisition of resistance to endoH digestion was monitored as the loss of intensity of the product of endoH digestion relative to the product of endoF digestion.

As shown in Fig. 2A, both WT and R402Q tyrosinase, expressed in cells grown at either 31 °C or 37 °C, were initially synthesized as ~72-kDa glycoproteins that are completely digested to ~55–60-kDa forms with either endoH or endoF. This is consistent with glycosylation at five of the six consensus sites and localization to the ER during the pulse period; the endoF product migrates slightly faster than the endoH product most likely due to the ~1 kDa of mass contributed from the N-acetyl glucosamine residues remaining after cleavage with endoH but not with endoF.

In cells grown at 37 °C, WT tyrosinase was degraded with a half-life of approximately 2.5 h, after an initial lag of about 1 h (Fig. 2, A and B). It was also slowly converted to an endoH-resistant form after a 1-h lag, concomitant with decreased mobility and increased heterogeneity by SDS-PAGE, indicating that some of the tyrosinase was exported from the ER before degradation. This was confirmed by the partial block in degradation of endoH-resistant tyrosinase by the addition of the lysosomotropic agents, MME or ammonium chloride, to the chase medium (Fig. 3A). The remainder of tyrosinase was likely degraded in a pre-Golgi compartment (see below). The overall degradation rate was reduced approximately 2-fold when cells

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In cells grown at 37 °C, WT tyrosinase was degraded with a half-life of approximately 2.5 h, after an initial lag of about 1 h (Fig. 2, A and B). It was also slowly converted to an endoH-resistant form after a 1-h lag, concomitant with decreased mobility and increased heterogeneity by SDS-PAGE, indicating that some of the tyrosinase was exported from the ER before degradation. This was confirmed by the partial block in degradation of endoH-resistant tyrosinase by the addition of the lysosomotropic agents, MME or ammonium chloride, to the chase medium (Fig. 3A). The remainder of tyrosinase was likely degraded in a pre-Golgi compartment (see below). The overall degradation rate was reduced approximately 2-fold when cells
were grown at 31 °C, and the time-dependent increase in the fraction of endoH-resistant material was much more dramatic, such that by 4 h, the majority of WT tyrosinase was endoH-resistant. These data support a slow release of WT tyrosinase from the ER at 37 °C and transport to late endosomes and lysosomes, where it was ultimately degraded; the rate of ER exit was enhanced by growth of cells at 31 °C, whereas the rate of degradation may be reduced.

In contrast to WT tyrosinase, endoH-resistant R402Q tyrosinase was almost never observed in cells grown at 37 °C (Fig. 2, A and C). The intensity of the endoF-sensitive band is nearly identical to that of the endoH-sensitive band at all time points, suggesting that R402Q tyrosinase does not progress in the secretory pathway beyond the cis Golgi. Furthermore, the loss of precipitable R402Q tyrosinase over time was largely insensitive to lysosomotropic reagents (Fig. 3A), suggestive of prelyosomal degradation. These data are consistent with the localization of this variant to the ER as observed by IFM. On the other hand, when cells were grown at 31 °C, a significant fraction of the R402Q tyrosinase became endoH-resistant (Fig. 2, A and C). This is less than the fraction of endoH-resistant WT tyrosinase by 8 h of chase at 31 °C, but nevertheless a dramatic increase relative to R402Q at 37 °C. Release of R402Q from the ER at 31 °C and subsequent passage through the Golgi complex to late endosomes and lysosomes, as evidenced by the degradation of the endoH-resistant fraction (Fig. 2, A and B), is consistent with the observed vesicular localization of this isoform by IFM. The results support an inability of R402Q tyrosinase to exit the ER at 37 °C rather than enhanced lysosomal degradation.

**Lack of a Significant Enhancement of Proteasomal Degradation of the R402Q Variant at the Restrictive Temperature**—The results thus far imply that the R402Q mutant fails to exit the ER at the restrictive temperature. ER retention is associated with incomplete or aberrant folding and consequent recognition of misfolded polypeptides by the ER quality control system (22, 23). Misfolded polypeptides also may serve as substrates for rapid degradation by the proteasome, which is thought to occur predominantly by reverse translocation to the cytosol and proteolysis by the proteasome (24, 40). Tyrosinase has been shown to be a substrate for this pathway in melanoma cells (41), and its degradation by this pathway is enhanced in some amelanotic melanoma cells (42). Thus, the apparent ER retention of R402Q tyrosinase at 37 °C may reflect enhanced proteasomal degradation.

To determine whether R402Q is degraded more rapidly than
WT tyrosinase by the proteasome, transfected HeLa cells grown at 31 °C or 37 °C were treated for various times with the proteasome inhibitors MG132 or lactacystin. Cells at each time point were harvested, and total cell lysates were analyzed by Western blotting with antibodies to tyrosinase. Treatment of cells expressing WT tyrosinase with MG132 resulted in the time-dependent formation of a band with an apparent molecular mass of 55 kDa. This band was not present in control lysates (Fig. 4A). A decrease in the intensity of this band was observed in lysates of cells treated with MG132 or lactacystin, and this decrease was more pronounced in cells grown at 37 °C than in cells grown at 31 °C (Fig. 4A, B). These results demonstrate that the WT tyrosinase is degraded by the proteasome in a time- and temperature-dependent manner.

**Temperature-sensitive Tyrosinase Retained in ER**

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**The Lumenal Domain of the R402Q Variant Is Sufficient to Mediate Temperature-sensitive ER Retention**—The position of the R402Q polymorphism within tyrosinase implicated the luminal domain of this variant as responsible for the ts ER retention phenotype (5). To confirm this and to determine whether other domains contribute to the ts phenotype, we constructed chimeric proteins in which different topologic domains of WT or R402Q tyrosinase were swapped with the corresponding domains of a marker protein, Tac. Tac (the human interleukin 2 receptor α chain), like tyrosinase, is a type I integral membrane glycoprotein. It is normally expressed at the cell surface and is efficiently exported from the ER (44). We previously showed that a chimeric protein with the luminal and transmembrane domains of Tac and the cytoplasmic domain of tyrosinase (TTY) accumulated in late endosomes and lysosomes and not the ER (39); since the cytoplasmic domains of WT and R402Q tyrosinase are identical, this excludes the possibility that the cytoplasmic domain contains an ER retention determinant. A series of chimeric proteins containing other combinations of tyrosinase and Tac topological domains, tagged at the C terminus with the HA11 epitope, was then constructed as outlined in Fig. 5 (top). Chimeric proteins were transiently expressed in HeLa cells, and localization was assessed in cells grown at 37 °C or 31 °C by IFM. To enhance the distinction between ER retention due to the ts defect versus the WT protein, cells were pretreated for 2 h with CHX to block protein synthesis, which results in clearance of WT tyrosinase from the ER (39).

**TYT-HA**

TYT-HA, containing only the transmembrane domain of tyrosinase, was expressed at the cell surface of transfected cells grown at either 31 °C or 37 °C (Fig. 5, i and j); this staining was similar to the control, TTT-HA, containing intact Tac with an HA11 epitope tag (data not shown; see Ref. 45). This shows that the tyrosinase transmembrane domain contains no information for ER retention. As expected, staining of cells transfected with WT/YT-HA (Fig. 5, a and b), containing the luminal and transmembrane domains of WT tyrosinase, or WT/YT-TT-HA (Fig. 5, c and f), containing the luminal domain only, fail to show extensive steady state ER localization at either temperature. In contrast, YT/YT-HA (Fig. 5, c and d) and YT/YT-TT-HA (Fig. 5, g and h), both containing the luminal domain of R402Q tyrosinase, are restricted to the ER in transfected cells grown at 37 °C but not in cells grown at 31 °C. The phenotype of Y/Y-TT-HA with the heterologous Tac transmembrane and cytoplasmic domains shows that, within the context of an integral membrane protein, the luminal domain of R402Q tyrosinase is sufficient for ts ER retention.

Curiously, neither Y/W-TT-HA nor Y/W-TT-TT-HA are delivered to the cell surface at 31 °C. Upon exiting the ER, these proteins appear to accumulate in the region of the Golgi complex. We do
ER Retention of the R402Q Variant Is Not Dependent on Tethering to the Membrane—ER quality control operates on both integral membrane proteins and soluble proteins, but different mechanisms may control each (46). To determine whether tethering to the membrane is either necessary for recognition of the R402Q variant as a substrate for ER quality control or influences the balance between retention and degradation, we examined the fate of a soluble form of tyrosinase. The transmembrane and cytoplasmic domains were deleted from both WT and R402Q tyrosinase, and a polyhistidine tag was fused to the C terminus of the luminal domain to allow easy purification of the transgene product. Tagged luminal domains were expressed transiently in HeLa cells. Given the apparent lack of post-ER targeting information in the WT tyrosinase luminal domain (see above), we expected these soluble forms to be secreted soon after exit from the ER. Thus, both cell lysates and supernatants from transfected cells grown at 31 °C or 37 °C were analyzed by Western blotting for the tyrosinase luminal domain. Samples were mock-treated or treated with endoH and endoF before SDS-PAGE to facilitate analyses.

As shown in Fig. 6, only endoH-sensitive forms of either soluble WT or R402Q tyrosinase accumulate in cell lysates (“cell”) from transfected cells grown at either 37 °C or 31 °C. This suggests that any material that progresses beyond the cis Golgi is either rapidly degraded or secreted from the cells. Supernatants (“sup”) of cells grown at 37 °C contain a small amount of soluble WT tyrosinase, amounting to less than 5% of the total expressed material at steady state (see the Fig. 6 legend). All of this material, however, is endoH-resistant, indicating proper passage through the secretory pathway. In contrast, essentially no material can be precipitated from supernatants of cells expressing soluble R402Q tyrosinase grown at 37 °C. This indicates that secretion of soluble R402Q is blocked at the restrictive temperature. The amount of endoH-resistant, soluble WT tyrosinase is dramatically increased in supernatants from cells grown at 31 °C, confirming the stabilizing effect of reduced temperatures on even the WT protein. Importantly, endoH-resistant, soluble R402Q is also recovered from supernatants of cells grown at 31 °C. These results show that the R402Q luminal domain alone exhibits the ts ER export phenotype and that tethering of the luminal domain to the membrane is not necessary for recognition by the ER quality control system. Furthermore, the results confirm the partial ts

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**Fig. 5.** The luminal domain of R402Q tyrosinase is sufficient to confer temperature-sensitive ER exit. **Top,** schematic diagram of Tac tyrosinase chimeric proteins. The luminal, transmembrane (TM), and cytoplasmic (Cyt) domains of tyrosinase, Tac, and chimeric constructs are represented schematically. Tyrosinase domains are represented in gray, and Tac domains and the HA11 epitope tag (HA) are represented in white. Names of the schematized proteins are indicated (top). **Bottom,** HeLa cells were transiently transfected with the indicated chimeric proteins, incubated at the indicated temperatures for 2 days, and treated with 50 μg/ml CHX for 2 h before fixation. Fixed cells were stained with a rabbit anti-calnexin antiserum and with a monoclonal antibody to the HA11 epitope, followed by fluorescein isothiocyanate- and lissamine-rhodamine-conjugated secondary antibodies; shown are only the images obtained with the anti-HA11 antibody.

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**Fig. 6.** Temperature-sensitive secretion of a soluble form of the R402Q tyrosinase luminal domain. Control pCDM8.1 plasmid (Vector; lanes 13–18) or plasmids encoding polyhistidine-tagged, soluble forms of the WT (lanes 1–6) or R402Q (lanes 7–12) tyrosinase luminal domain, in which the transmembrane and cytoplasmic domains were deleted, were transiently transfected into HeLa cells. Transfected cells were then grown at the indicated temperature. Two days post-transfection, cells and culture supernatants were harvested. Cell lysates (cell; 5% of total from the experiment; lanes 1–3, 7–9, and 13–15) or nickel-agarose-precipitated culture supernatants (sup.; 35% of total from the experiment; lanes 4–6, 10–12, and 16–18) were mock (−), endoH (+), or endoF (F)-treated (endo tmt.) as indicated and then separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride, probed with NY8K anti-tyrosinase serum, and detected by ECL. Migration of molecular mass standards (kDa) is indicated to the right.
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Fig. 7. Temperature-sensitive misfolding of the R402Q tyrosinase lumenal domain is irreversible. Polyhistidine-tagged, soluble forms of the WT or R402Q tyrosinase lumenal domain, in which the transmembrane and cytoplasmic domains were deleted, were transiently transfected into HeLa cells. Cells were then grown at the indicated temperature (initial temp.) for 2 days. Two days post-transfection, the culture medium was replaced with medium containing 10 μg/ml CHX. Cells were incubated for 30 min at the initial temperature and then were either harvested (panels A and C) or shifted to the final temperature (final temp.) and grown for 7 h in fresh medium supplemented with CHX, at which point both cells and culture supernatants were harvested (panels B and D). Cell lysates (cell; 5% of total from the experiment) and nickel-agarose-precipitated culture medium (sup.; 50% of total from the experiment) were mock (−), or endoH (+)-treated and then separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride, probed with NY8K anti-tyrosinase serum, and detected by ECL. Migration of molecular mass standards (kDa) is indicated to the right.

**DISCUSSION**

Ts allelic isoforms of tyrosinase are abundant throughout the animal kingdom (2, 8, 9, 48, 49), but the molecular basis for their ts phenotype has not been characterized. Our data demonstrate that the ts enzymatic activities of the common R402Q allele of human tyrosinase (10) are explained by nearly absolute and irreversible ER retention at the restrictive temperature. Growth at 31 °C permits ER exit of at least a fraction of newly synthesized tyrosinase and subsequent transport to late endosomes or melanosomes. Ts ER retention is a feature of other known ts protein variants in the mammalian secretory pathway (15–19), and it is likely that other ts allelic forms of tyrosinase share this phenotype. Indeed, ts ER retention of tyrosinase has been observed in metastatic amelanotic melanoma cells (42); these tumor cells may have either accumulated similar mutations during the development of metastases or derived from donors expressing a ts allelic form of tyrosinase. Taken together, our results imply that ER quality control may be a central mechanism for governing variable pigmentation and vision throughout the animal kingdom.

The mechanism underlying ER retention of tyrosinase at the restrictive temperature is most likely a defect that prevents competent folding. The quality control machinery of the ER functions to assure that misfolded polypeptides are retained in the ER before ultimate degradation (20, 23). Pathogenic ts mutants of the cystic fibrosis transporter (16) and the Wilson’s copper transporter (15), causative agents in cystic fibrosis and Wilson’s disease, respectively, are similarly misfolded and re-

**Temperature-sensitivity Phenotype of the R402Q Variant Is Irreversible**—The ts folding and ER retention phenotype of R402Q tyrosinase is similar to that of several other ts glycoproteins. In some cases, such as with the ts045 variant of the vesicular stomatitis virus glycoprotein, the ts phenotype is reversible such that misfolded polypeptides that accumulate at the restrictive temperature can be rescued by a subsequent shift to the permissive temperature (47). Rescue may be indicative of a partially folded intermediate that has not entered a “dead-end” misfolded pathway. In other examples, misfolded polypeptides become irreversibly trapped in an unfolded state and cannot be rescued (20). The placement into one category or the other may facilitate understanding of the manner in which misfolded polypeptides are recognized and handled by the quality control machinery of the ER.

To determine whether the R402Q variation imparted a reversible or irreversible misfolding event, we took advantage of the ability to capture fully folded, transport-competent lumenal domain by secretion of the soluble form. HeLa cells transfected with either soluble WT or soluble R402Q tyrosinase were grown at 37 °C for 48 h and then incubated with CHX for 30 min to block further protein synthesis of new tyrosinase molecules. Cells were then either maintained at 37 °C or shifted to 31 °C, and the medium was replaced. CHX was maintained in the medium to limit analyses to only those molecules that had accumulated at the nonpermissive temperature before the temperature shift. Cell lysates and supernatants were then assayed after 7–8 h for the presence of soluble tyrosinase in the medium. Control cultures show that easily detectable levels (5% of total in a parallel experiment) of soluble R402Q tyrosinase were recovered in the medium when cells are initially grown and then maintained at 31 °C during the chase (Fig. 7D, lanes 23–26). However, only trace amounts are detected in supernatants of cells that are grown at 37 °C before the 31 °C chase (Fig. 7D, lanes 19–22; 1% of total in a parallel experiment, relative to 0.4% of total from cells maintained at 37 °C throughout the experiment). Thus, soluble R402Q tyrosinase that is irreversibly misfolded at 37 °C cannot be rescued by a subsequent chase at the permissive temperature. Although the small amount of material detected in the medium after the 31 °C chase may represent a fraction of soluble R402Q tyrosinase that is capable of refolding, it more likely represents material synthesized during the chase, since the low level of CHX used, necessary to maintain viability, only blocked 80% of protein synthesis (data not shown). In contrast to the R402Q variant, soluble WT tyrosinase is recovered in the medium when the initial cultures are maintained at 37 °C regardless of the chase temperature; however, consistent with the pulse-chase analyses in Fig. 2, significantly more soluble WT tyrosinase is recovered when the chase is performed at 31 °C (6% of total in a parallel experiment) than at 37 °C (3.5% of total; Fig. 7B, compare lanes 5 and 6 with 9 and 10). This indicates that WT tyrosinase, unlike the R402Q variant, does not irreversibly misfold at 37 °C and can be rescued by a subsequent reduction in growth temperature.
tained in the ER at the restrictive temperature but fold sufficiently to bypass ER quality control at reduced temperatures. Non-mutant proteins that fold inefficiently, such as the cystic fibrosis transporter (50) and peptide-free major histocompatibility complex class I molecules (51), may also escape quality control and be exported from the ER at reduced temperatures, as we have shown here for WT tyrosinase. We have observed similar results for endogenous tyrosinase expressed in a number of highly pigmented melanoma cell lines, suggesting that this is an intrinsic property of human tyrosinase. We suspect that WT tyrosinase enters a partially folded intermediate at 37 °C that can be stabilized at lower temperatures, because soluble WT tyrosinase molecules that had accumulated at 37 °C could be released and secreted by a subsequent shift to 31 °C. In contrast, the R402Q variant could not be released under these conditions. Perhaps the R402Q variation stabilizes or favors a nonproductive intermediate that occurs normally for tyrosinase, eventually leading to entrapment in a stable misfolded state (14). Stringent kinetic analyses of the ability to rescue misfolded WT or R402Q tyrosinase at 31 °C and of disulfide bond formation may help to test this model. Unfortunately, its inability to be rescued by a post-synthetic reduction in temperature makes R402Q tyrosinase untenable for use as a synchronous marker of biosynthetic transport in the manner that the ts045 variant of the vesicular stomatitis virus glycoprotein has been used (47).

Using Western blotting of cell lysates treated for increasing time periods with specific inhibitors, both WT and R402Q isoforms of tyrosinase were shown to be substrates for degradation by the proteasome. Protected fragments similar in size to deglycosylated full-length tyrosinase molecules accumulated in the presence of the inhibitors. These results support earlier studies demonstrating proteasomal degradation of tyrosinase (41, 42). Despite the differences between WT and R402Q in ER retention, there appeared to be little difference in the rate of accumulation of proteasome-protected fragments. Indeed, the persistence of endoH-sensitive tyrosinase up to 4 h of chase indicates that the rate of tyrosinase degradation by this pathway is slow, and thus, only a fraction of ER-retained WT or R402Q tyrosinase is degraded by this pathway at any given time. These results suggest that the ER quality control system distinguishes between different misfolded states of tyrosinase, targeting some molecules (perhaps those more severely misfolded) for rapid retrotranslocation and proteasomal degradation while permitting others (perhaps those with more subtle folding defects or partially folded intermediates) to remain in the ER lumen. Our data suggest that similar fractions of WT and R402Q tyrosinase achieve a state recognized as a substrate for proteasomal degradation. The remaining molecules serve as substrates for ER retention, giving the WT tyrosinase an opportunity to properly fold and exit the ER. Eventually, all tyrosinase unable to correctly fold is degraded by the proteasome and/or by additional proteolytic systems.

Because the amino acid variation R402Q is positioned within the luminal domain, it was not surprising that membrane-tethered chimeric proteins containing the R402Q tyrosinase luminal domain displayed ts ER retention. Perhaps more surprising was the finding that the luminal domain alone, expressed as a soluble fragment, was also ts for ER retention. This suggests that the mechanisms that operate to retain this variant within the ER are independent of the membrane association of the substrate. Such flexibility poses an interesting conformational problem, since membrane-bound tyrosinase is restrained to move only in the plane of the membrane, whereas soluble tyrosinase should be free to diffuse throughout the ER lumen once translation is completed. Thus, either these proteins are recognized in a manner independent of topological constraints, there are redundant mechanisms to effect ER retention, or retention mechanisms initiate during protein translocation while the soluble form is still tethered to the membrane. Calnexin, an ER membrane-bound lectin-like chaperone protein, is a major participant in ER retention of misfolded, unassembled, and partially folded integral membrane and soluble polypeptides, (reviewed in Ref. 23; see also Refs. 52 and 53) and likely contributes to ER retention of membrane-tethered R402Q tyrosinase and of soluble tyrosinase during translation. Calnexin has been reported to be stably associated with a large fraction of tyrosinase in melanocytes and melanoma cells (42, 54, 55) and to be required for proper tyrosinase folding in COS-7 cells (56).

WT tyrosinase that exits the ER in HeLa cells appears to be targeted for degradation in late endosomes and lysosomes, as suggested by the loss of endoH-resistant material during long chase times in pulse-chase experiments, the sensitivity of this loss to ammonium chloride and MME, and the localization of tyrosinase to late endosomes and lysosomes in HeLa cells at steady state. By contrast, tyrosinase in cells of the melanocyte lineage is reported to be highly stable (57–59). At least two potential explanations may account for this discrepancy. First, tyrosinase may be inappropriately targeted to a degradative compartment in HeLa cells but not in pigmented melanocytic cells. This is supported by the segregation of tyrosinase-positive compartments from the bulk of lampl1-positive late endosomes and lysosomes in melanoma cells but not in HeLa cells (data not shown and Ref. 60). Second, HeLa cells may lack a stabilizing factor for tyrosinase in late endosomal compartments. This is supported by the lower stability of murine tyrosinase in a melanocyte cell line lacking expression of the tyrosinase-related protein TRP1 relative to a melanocyte line from WT mice (57). Further analyses of the expression of tyrosinase in pigmented versus nonpigmented cells will be necessary to assess these two mechanisms.

Curiously, lysosomotropic reagents protected not only endoH-resistant tyrosinase from degradation but also endoH-sensitive tyrosinase in a pulse-chase experiment (see Fig. 3, A and B). This could potentially be explained by autophagic degradation of aggregates of misfolded tyrosinase that had accumulated in regions of the ER, such as “aggresomes” (61). Additional experiments would be required to test this hypothesis and to determine whether this reflects a physiological degradation pathway or is merely due to overexpression of misfolded tyrosinase in transfected cells.

What do our results imply for the etiology and treatment of OCA1? There are numerous missense mutations within the gene for tyrosinase that result in OCA1, many of them clustering around the coding region for the putative CuB copper binding site near residue 402. Given the inefficient folding of WT tyrosinase and the further defect in folding of the R402Q variant, it is likely that many of these mutations do not affect enzyme activity per se but rather cause the protein to be misfolded and retained and/or degraded in the ER. The accelerated degradation of tyrosinase observed in amelanotic melanoma cells (42) is likely due to a similar phenomenon in which these cells accumulated mutations that further decrease the fidelity of tyrosinase folding. If such speculation is validated, pharmacological intervention targeted toward enhancing productive folding of tyrosinase in the ER may provide a common method to enhance pigmentation in OCA1 patients carrying distinct mutations in tyrosinase. Further analysis of the effects of enhanced chaperone expression, decreased cell temperature, or

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2 D. W. Frank and J. F. Berson, unpublished information.
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cell treatment with stabilizing agents such as glycerol (50) on the ability to export various mutants of tyrosinase would help to determine whether our findings extend to other OCA1 mutants.

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