The molecular basis of oculocutaneous albinism type 1 (OCA1): sorting failure and degradation of mutant tyrosinases results in a lack of pigmentation

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Oculocutaneous albinism type 1 (OCA1) is an autosomal recessive disease resulting from mutations of the tyrosinase gene (*TYR*). To elucidate the molecular basis of OCA1 phenotypes, we analysed the early processing and maturation of several different types of mutant tyrosinase with various degrees of structural abnormalities (i.e. two large deletion mutants, two missense mutants that completely destroy catalytic function and three missense mutants that have a temperature-sensitive phenotype). When expressed in COS7 cells, all mutant tyrosinases were sensitive to endoglycosidase H digestion, and immunostaining showed their localization in the endoplasmic reticulum (ER) and their failure to be sorted further to their target organelles. Pulse–chase experiments showed that all mutant tyrosinases were retained by calnexin in the ER and that they were degraded at similarly rapid rates, which coincided with their dissociation from calnexin. Temperature-sensitive mutant enzymes were sorted more efficiently at 31 °C than at 37 °C, and their degradation was accelerated at 37 °C compared with 31 °C. Thus in contrast to the current concept that mutant tyrosinases are transported to melanosomes but are functionally inactive there, our results suggest that mutant tyrosinases may not be transported to melanosomes in the first place. We conclude that a significant component of mutant tyrosinase malfunction in OCA1 results from their retention and degradation in the ER compartment. This quality-control process is highly sensitive to minimal changes in protein folding, and so even relatively minor mutations in peripheral sequences of the enzyme not involved with catalytic activity may result in a significant reduction of functional enzyme in melanosomes.

Key words: calnexin, mutant protein, protein transport.

INTRODUCTION

Oculocutaneous albinism (OCA) is the name for a group of severe genetic disorders of pigmentation characterized by reduced or absent biosynthesis of melanin pigment in melanocytes of the skin, hair follicles and eyes [1,2]. OCA type 1 (OCA1) is an autosomal recessive disorder resulting from the deficient catalytic function of tyrosinase (monophenol mono-oxygenase, EC 1.14.18.1). Tyrosinase is normally transported to melanosomes, specialized organelles in which melanin is synthesized and deposited on their internal matrix. Catalytic activity of tyrosinase is absolutely necessary for pigment production, and two copper ions that bind at two distinct regions, termed CuA and CuB, are required for its catalytic function. The classic concept of the OCA phenotype has been that mutant tyrosinases are sorted to melanosomes but are catalytically inactive there.

In humans, at least two different subtypes of OCA1, termed OCA1A and OCA1B, have been distinguished clinically. In OCA1A (formerly known as 'tyrosinase-negative OCA'), the classic and most severe form of the disorder, tyrosinase activity is completely absent and melanin pigment cannot be detected in the skin, hair or eyes [3]. A total of 78 distinct mutations of the tyrosinase gene (*TYR*), including 48 missense substitutions, have been reported to date in OCA1A [4]. In OCA1B (formerly termed 'yellow OCA'), tyrosinase activity and melanin synthesis are greatly reduced. Some melanization may occur during childhood and adult life, although little or no melanin pigmentation is evident at birth. Ten distinct mutations of the *TYR* gene, including nine missense substitutions, have been reported in OCA1B [4]. In particular, the R402Q, P406L and R422Q substitutions result in tyrosinases with temperature-sensitive catalytic activities that are abolished at 37 °C and are only weakly expressed at 31 °C *in vivo* [5] and *in vitro* [6,7].

It is of great interest to understand how such a wide variety of mutations, even those with very mild predicted effects on enzyme structure and function, often have such dramatic effects on phenotype and why some of them are temperature-sensitive. OCA1 missense mutations tend to be located in four clusters in the tyrosinase polypeptide [1,2], and computer modelling has suggested that some of these may alter the folding of tyrosinase, disrupting association of the two copper ions and thereby destroying catalytic function [8]. We have shown previously that the ability to bind copper is essential for correct folding of tyrosinase and expression of its enzyme activity, and that several mutations abolish or reduce this ability [9]. Surprisingly, one temperature-sensitive mutant tyrosinase, P406L, was found to bind copper normally but expressed no tyrosinase enzyme activity when incubated at 37 °C [9], and we have so far been unable to explain that paradox.

Defective transport and processing of proteins can result in their retention in the endoplasmic reticulum (ER) [10,11], the location of the so-called 'quality-control system' by which

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; endo H, endoglycosidase H; ER, endoplasmic reticulum; OCA, oculocutaneous albinism.

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proteins that fail to be properly folded are not transported to their final destinations but instead are degraded by proteolysis [12–16]. Some of those proteins interact transiently in the ER with a molecular chaperone, such as calreticulin [16] or calnexin [12–14], a lectin-like chaperone protein that binds specifically to N-linked oligosaccharide processing intermediates of newly synthesized proteins [17,18]. Such interactions reduce the rate of ubiquitin-dependent degradation of misfolded glycoproteins [19,20].

As recent studies from a number of laboratories have shown that post-translational glycosylation and calnexin processing are critically involved in the maturation and function of tyrosinase ([21–26] and just reviewed in [27]), we hypothesized that mutant tyrosinases associated with various subtypes of OCA1 might as a general rule be retained in the ER and then degraded, rather than being transported to melanosomes. To test this hypothesis, we constructed a series of tyrosinase cDNA expression vectors containing a number of distinct OCA1A and OCA1B mutations, as well as tyrosinase cDNA deletions that lack the CuA or CuB copper-binding domains. By systematic analysis of the processing and maturation of the corresponding tyrosinase polypeptides, we conclude that all mutant tyrosinases examined manifest prolonged association with calnexin and are subsequently degraded upon dissociation from it. This new understanding greatly enhances our concept of the aetiology of OCA1, and explains a number of seemingly paradoxical earlier findings.

EXPERIMENTAL

Cells and antibodies used

COS7 (African green monkey kidney) cells (CRL 1650, American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Gibco-BRL, Rockville, MD, U.S.A.). Calnexin polyclonal antibody (SPA-860) was from StressGen (Victoria, Canada), the mouse monoclonal human tyrosinase antibody (NCL-TYROS) was from Novocastra Laboratories (Newcastle upon Tyne, U.K.), and the αPEP7h rabbit antiserum to tyrosinase was raised in our laboratory against residues 513–529 of human tyrosinase [28]. Alexa 595-labelled goat anti-mouse IgG [$F(ab)_2$] and Alexa 488-labelled goat anti-rabbit IgG [$F(ab)_2$] were from Molecular Probes (Eugene, OR, U.S.A.). Anti-rabbit Ig horseradish peroxidase-linked whole antibody was from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

Proteinase inhibition

The proteasome inhibitors, MG132 and lactacystin (Calbiochem, San Diego, CA, U.S.A.), the cysteine proteinase inhibitor E64, and the serine proteinase inhibitor PMSF (Roche, Indianapolis, IN, U.S.A.) were dissolved in DMSO to make a 1000 × stock solution; 50 μM for MG132, 20 μM for lactacystin, 50 μM for E64 and 1 mM for PMSF. COS7 cells transfected with tyrosinase plasmids were pre-incubated with DMEM containing 0.1 % DMSO, E64, PMSF, MG132 or lactacystin for 6 h prior to radiolabelling with [^{35}S]methionine for 20 min. These components were also added during the radiolabelling and chase periods.

Expression vectors and transfection techniques

All constructs were based on the normal human tyrosinase cDNA expression plasmid, pcTYR [29]. Plasmids pcTYR-A206T, pcTYR-T373K, pcTYR-Δ19-159, pcTYR-Δ316-443, pcTYR-R402Q, pcTYR-P406L and pcTYR-R422Q were as described previously [6,9]. COS7 cells were transiently transfected using LipofectAMINE (Gibco-BRL). Briefly, cells were grown to 50–60 % confluence in 35 mm dishes for 16 h before transfection, and 0.5 μg of each expression vector was mixed with 2 μl of LipofectAMINE in 1 ml of OPTI-MEM reduced-serum medium (Gibco BRL). COS7 cells were incubated with the transfection mixture for 5 h, washed three times with DMEM, incubated in fresh DMEM containing 10 % fetal calf serum in a 5 % CO_2 atmosphere for 48 h, and then harvested.

Immunohistochemical staining

Transfected cells were plated in 4-well Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL, U.S.A.) 24 h before each experiment, and were then fixed in PBS containing 3.7 % formaldehyde for 20 min at 23 °C, and subsequently permeabilized with methanol for 20 min at 4 °C. All subsequent processing was done at 23 °C. Cells were blocked in PBS containing 0.5 % BSA and 50 mM Tris/HCl, and were then washed three times in the same buffer to wash out the antibody after each step. Cells were first incubated with mouse monoclonal anti-human tyrosinase (NCL-TYROS; 1:200) for 1 h followed by incubation with Alexa 595-labelled goat anti-mouse IgG [$F(ab)_2$] (1:400), and then incubated with anti-calnexin antibody (1:400). Slides were then incubated with Alexa 488-labelled goat anti-rabbit IgG [$F(ab)_2$] (1:400). The stained slides were observed and photographed with a Leica DMR fluorescence microscope (Leica, Wetzlar, Germany).

Western blotting and endoglycosidase H (endo H) digestion

For Western blotting, cells were solubilized for 1 h at 4 °C in 150 mM NaCl, 0.5 % deoxycholic acid, 1 % Nonidet P40, 0.1 % SDS and 50 mM Tris/HCl, pH 7.5, containing protease-inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). After protein concentrations were measured and equalized, lysates containing 0.5 μg (2.5 μl) of protein each were resuspended in 2.5 μl of 50 mM sodium acetate, 0.5 % SDS and 1 % 2-mercaptoethanol, pH 5.5, and heated at 95 °C for 10 min. Sodium acetate (5 μl, 50 mM), pH 5.5, with or without 0.25 unit of endo H (Boehringer Mannheim) was added to each sample and incubated for 3 h at 37 °C. Endo H digestions were terminated by adding 15 μl of 0.9 M Tris/HCl, pH 8.45, 24 % (v/v) glycerol, 8 % SDS, 0.015 % Coomassie Blue G and 0.005 % Phenol Red (Novex, San Diego, CA, U.S.A.) and heating at 95 °C. Samples were separated by SDS/PAGE (8 % gels; Novex) and were transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Blots were blocked in 5 % non-fat milk in 20 mM Tris, 137 mM NaCl, 3.8 mM HCl and 0.1 % Tween 20, pH 7.6 (TBS-Tween), for 2 h and were then incubated with αPEP7h (1:1000) in TBS-Tween. After four washes, blots were incubated in anti-rabbit horseradish peroxidase-linked whole antibody (1:1000) in TBS-Tween, and immunoreactivity was detected by ECL Western blotting detection (Amersham Pharmacia Biotech).

Metabolic labelling and immunoprecipitation

Metabolic labelling and immunoprecipitation experiments were performed as described previously [21] with some modifications. Briefly, transfected cells were cultured in methionine-depleted DMEM, then labelled at 37 °C for the times noted with [^{35}S]-methionine (30 μCi; Amersham Pharmacia Biotech). For pulse–chase experiments, cells were labelled for 20 min or for 60 min as noted, then chased for specific periods up to 20 h in DMEM

supplemented with 1 mM unlabelled methionine. Cells were harvested and then washed twice with PBS at 4 °C and solubilized for 1 h at 4 °C in 2% Triton-X 100, 400 mM KCl and 50 mM triethanolamine acetic acid, pH 7.2, containing protease-inhibitor cocktail. For immunoprecipitation, the clarified supernatants were preincubated with 20 μl of Pansorbin (Calbiochem) for 1 h at 4 °C with mixing. The supernatants were collected by centrifugation at 4 °C and incubated with tyrosinase (1:200) or calnexin (1:400) antibodies at 4 °C for 1 h with mixing. Pansorbin (20 μl) was then added to each sample, which were incubated at 4 °C with mixing for 1 h. The immune complexes were washed with 0.7 M KCl, 0.5 M NaCl, 0.05% Triton X-100 and 10 mM Tris/HCl, pH 7.5, by centrifugation. The final pellets were separated directly by SDS/PAGE, or were kept for further experiments, such as endo H digestion or sequential immunoprecipitation, as detailed below. For SDS/PAGE, the pellets were resuspended in 2 × SDS sample buffer, heated at 95 °C for 5 min, and centrifuged. The samples were separated by SDS/PAGE (8% gels) and the separated protein bands were by fluorography using the rapid autoradiography enhancer, EnLightning (NEN Life Science Products, Boston, MA, U.S.A.), and Kodak X-OMAT AR X-ray film (Eastman Kodak, Rochester, NY, U.S.A.).

For endo H digestion, immune complexes were resuspended in 5 μl of 50 mM sodium acetate, 0.5% SDS and 1% 2-mercaptoethanol, pH 5.5, and heated at 95 °C for 10 min. Sodium acetate (5 μl, 50 mM), pH 5.5, and 0.01 unit of endo H were added to each sample. Samples were incubated for 3 h at 37 °C and digestions were terminated by adding 15 μl of 2 × SDS sample buffer and heating at 95 °C for 5 min.

For sequential immunoprecipitation, immune complexes were resuspended in 20 μl of 1% SDS, 5 mM EDTA and 10 mM Tris/HCl, pH 7.5, and heated at 95 °C for 5 min. Supernatants were collected by centrifugation, and 80 μl of 2% Triton X-100, 0.15 M NaCl and 10 mM Tris/HCl, pH 7.5, were added to each. Samples were again preincubated with 20 μl of Pansorbin for 1 h at 4 °C with mixing. The collected supernatants were incubated with the second antibody, as described in the figure legends, for 1 h at 4 °C with mixing. Samples were again incubated with 20 μl of Pansorbin for 1 h at 4 °C with mixing to collect immune complexes. After centrifugation, the final pellets were resuspended in 2 × SDS sample buffer, heated at 95 °C for 5 min, separated by SDS/PAGE (8% gels), and visualized by fluorography as described above.

RESULTS

Effects of tyrosinase mutations on subcellular distribution

In normal melanocytes, wild-type tyrosinase is sorted to melanosomes; however, when tyrosinase is expressed by transfection in non-pigment cells it is sorted to lysosomes [21,30], which have a biogenesis pathway in common with melanosomes [31,32]. We showed previously that wild-type tyrosinase expressed in COS7 cells was localized in the ER and in lysosomes, and that co-expression of calnexin promoted the redistribution of tyrosinase from the ER to lysosomes [21]. We have also reported that COS7 cells transfected with plasmids encoding many mutant tyrosinases (A206T, T373K, Δ19-159, Δ316-443) have no functional tyrosinase enzyme activity at 37 °C or at 31 °C [6,9], whereas cells transfected with the temperature-sensitive mutant tyrosinases (R402Q, P406L or R422Q) have no enzymic function at 37 °C but regain much (40–50%) of their catalytic function at 31 °C [9]. In the present study, to investigate the subcellular localization of those mutant tyrosinases we transfected mutant tyrosinase expression plasmids into COS7 cells and used immunohistochemistry to compare the subcellular distribution of mutant tyrosinases with that of wild-type tyrosinase. As shown in Figure 1, normal tyrosinase (left-hand panels in red) expressed at 37 °C or 31 °C was localized in granules around the perinuclear rim and in a reticular pattern. That granular staining pattern is consistent with the sorting of wild-type tyrosinase to lysosomes, as we have shown previously [21]. Calnexin-staining patterns in those same cells (Figure 1, middle panels in green) had a similar reticular pattern of staining, which co-localized with tyrosinase in the ER (yellow in the superimposed images in the right-hand panels).

In contrast, mutant tyrosinases expressed in transfected COS7 cells at 37 °C exhibited staining patterns markedly different from normal tyrosinase. For example, A206T mutant tyrosinase showed only reticular staining, without any obvious particulate structures, a distribution pattern identical to calnexin. In fact, all mutant tyrosinases examined (A206T, T373K, Δ19-159, Δ316-443, R402Q, P406L and R422Q) showed that same reticular pattern when cultured at 37 °C and none of them had any granular component.

The subcellular distributions of most mutant tyrosinases (A206T, T373K, Δ19-159 and Δ316-443) were essentially unchanged when the transfected cells were cultured at 31 °C. However, the distributions of the temperature-sensitive mutant tyrosinases (R402Q, P406L or R422Q) revealed a partial rescue of sorting to particulate lysosomes at the more permissive temperature. Although the distributions of temperature-sensitive mutant tyrosinases expressed at 37 °C were completely reticular, when cultured at 31 °C their distribution patterns changed to a particulate pattern similar to wild-type tyrosinase.

Sensitivity of mutant tyrosinases to endo H

To confirm that mutant tyrosinases are not localized in lysosomes, we used Western blotting to assess their sensitivities to endo H, an enzyme that removes carbohydrate residues from N-linked glycoproteins prior to the late processing that occurs in the Golgi. As shown in Figure 2, in transfected COS7 cells cultured at 37 °C, wild-type tyrosinase was synthesized as a glycoprotein of 69–75 kDa, and upon treatment with endo H the majority of wild-type tyrosinase was sensitive and only a small amount was resistant. At 31 °C, the proportion of wild-type tyrosinase that was endo H-resistant was increased. In contrast, all mutant tyrosinases examined were completely sensitive to endo H when transfected cells were incubated at 37 °C and only the temperature-sensitive tyrosinases became partially resistant to endo H when the transfected cells were cultured at 31 °C. These data show clearly that mutant tyrosinases are retained in the ER at 37 °C and are not processed through the Golgi, confirming the immunohistochemical results presented above. Only the temperature-sensitive mutant tyrosinases were partially rescued when transfected cells were cultured at the more permissive temperature of 31 °C.

Association of calnexin with tyrosinase

To determine whether calnexin binds to newly synthesized tyrosinase, COS7 cells expressing wild-type tyrosinase were radiolabelled with [^{35}S]methionine, and labelled proteins were then immunoprecipitated with either normal rabbit serum or antibodies recognizing calnexin or tyrosinase (Figure 3). Sequential immunoprecipitation was also performed, i.e. proteins that had been precipitated with calnexin antibody were then immunoprecipitated again with normal rabbit serum, or with antibodies to calnexin or tyrosinase. Several proteins could be seen to co-

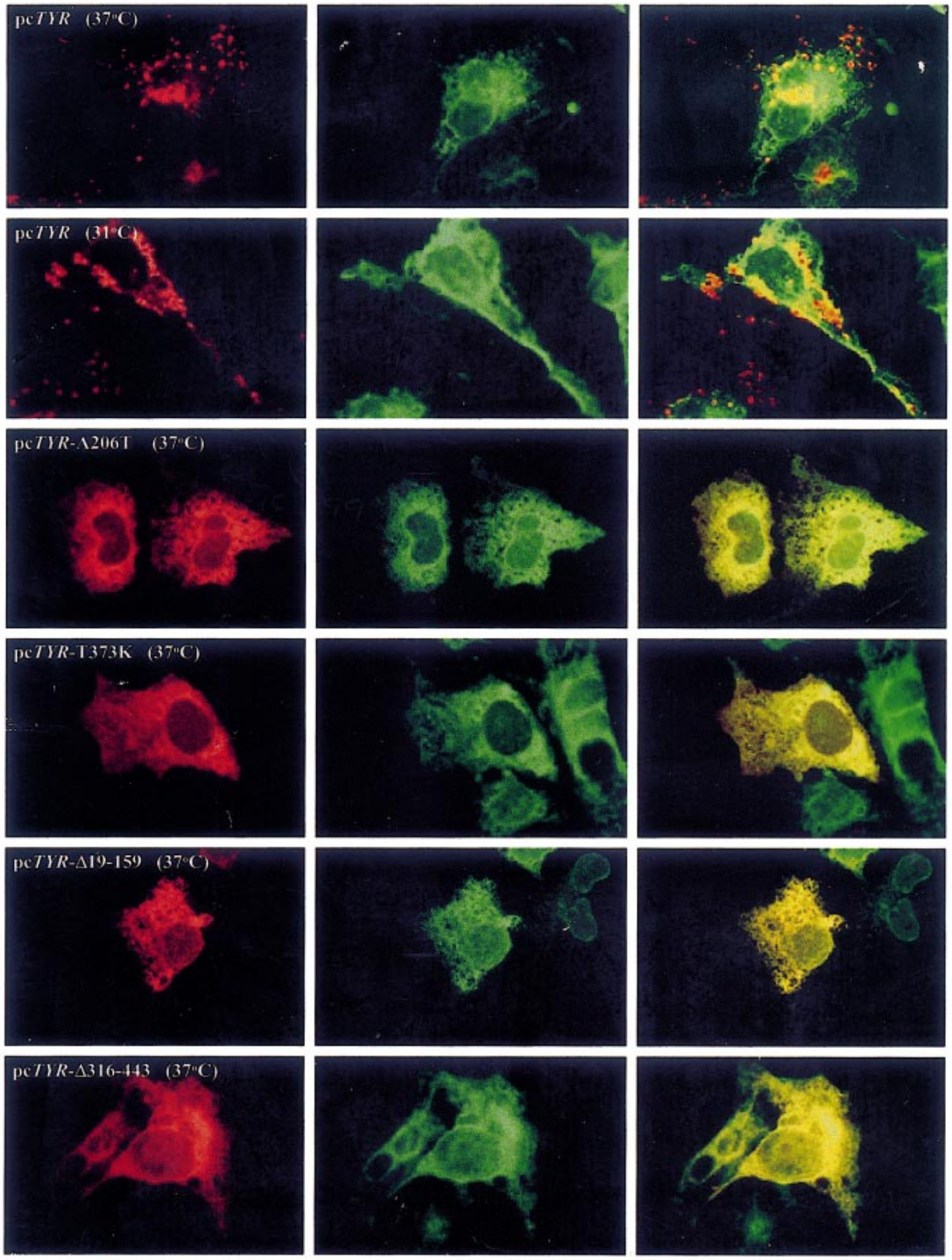

Figure 1 For legend see facing page.

precipitate with calnexin, and the second immunoprecipitation showed that the major band of 69–75 kDa corresponds to tyrosinase.

Degradation of mutant tyrosinases

Calnexin functions as a molecular chaperone in the maturation of tyrosinase, binding it and forming a transient calnexin–protein complex [21]. Binding of mutant tyrosinases with altered three-dimensional structures to calnexin would be expected to be prolonged. To demonstrate the physical interactions of wild-type or mutant tyrosinases with calnexin, we performed pulse–chase experiments of COS7 cells transfected with pcTYR, pcTYR-A206T, pcTYR-Δ19-159 or pcTYR-P406L. As shown in Figure 4, calnexin was detected in all transfected cells as an 88–90 kDa protein. Tyrosinases in the transfected cells were detected as

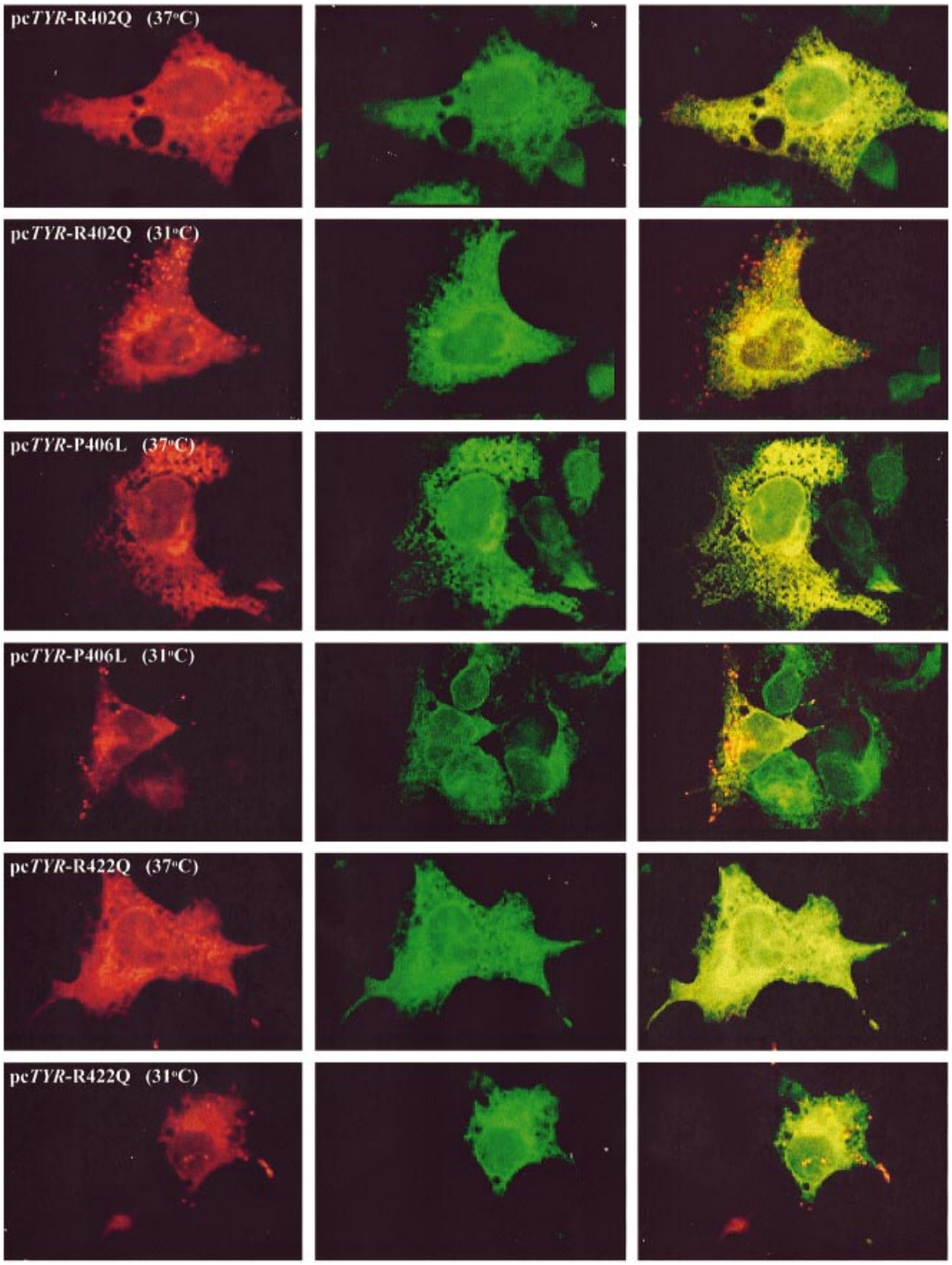

Figure 1 Subcellular localization of wild-type and mutant tyrosinases by immunohistochemistry

COS7 cells were transiently transfected with plasmids encoding wild-type tyrosinase (*TYR*), substitution mutant tyrosinases (A206T or T373K), truncated mutant tyrosinases (Δ19-159 or Δ316-443) or temperature-sensitive mutant tyrosinases (R402Q, P406L or R422Q). Transfected cells were cultured on coverslips at 37 °C or, where noted, at 31 °C. After fixation, the cells were incubated with the tyrosinase antibody and visualized by staining with Alexa 595-labelled goat-anti-mouse IgG (left-hand panels). They were then incubated again with calnexin antibody and visualized by staining with Alexa 488-labelled goat anti-rabbit IgG (middle panels). Superimposed pictures were taken using a filter (right-hand panels). The distribution of tyrosinase in pcTYR-A206T-, pcTYR-T373K-, pcTYR-Δ19-159- or pcTYR-Δ316-443-transfected cells was unchanged when cultured at 31 °C (results not shown).

doublets (one major band and one minor band) at 69–75 kDa, as expected, except for cells transfected with pc*TYR*-Δ19-159, in which the truncated form of tyrosinase was detected at the predicted smaller size, 55–60 kDa. In all cases the expressed tyrosinases were the major proteins that co-precipitated with calnexin, since relatively large amounts of tyrosinase are expressed in those transfected cells, although calnexin also formed complexes with various minor glycoproteins. The identity of

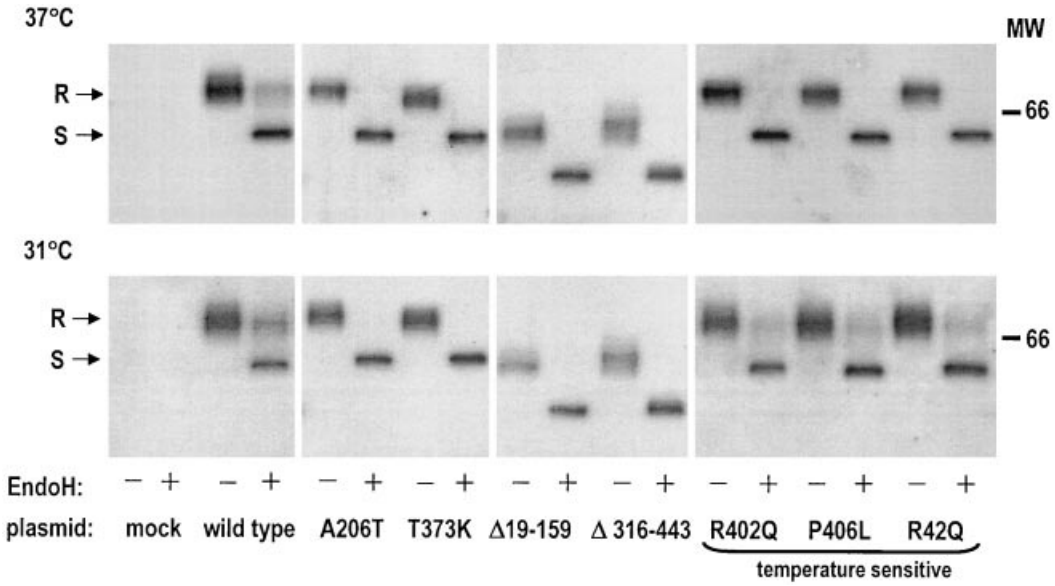

Figure 2 Endo H resistance of tyrosinases at 37 °C or 31 °C

COS7 cells were transiently transfected with plasmids and were cultured for 2 days at 37 °C (upper panel) or 31 °C (lower panel). The cells were solubilized and, after protein concentrations were measured and equalized, lysates each containing 0.5 μg of protein were incubated with (+) or without (−) endo H. Samples were then separated by SDS/PAGE and electrophoretically transferred to blotting membranes. Tyrosinases were visualized by staining with tyrosinase antibody and fluorography, as detailed in the Experimental section. R, endo H-resistant band; S, endo H-sensitive band. The sizes of the deletion-mutant tyrosinases are less than that of the wild-type enzyme, as discussed in the text.

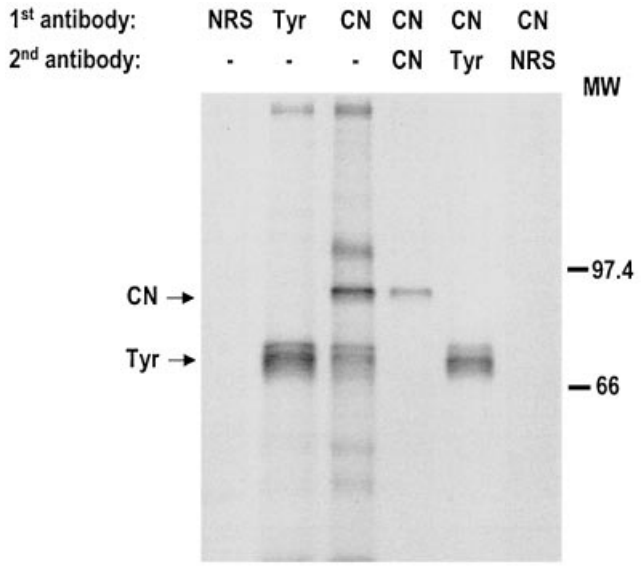

Figure 3 Association of calnexin with tyrosinase

COS7 cells transfected with wild-type tyrosinase were pulse-labelled with [^{35}S]methionine for 1 h at 37 °C. Cell lysates were centrifuged, and the supernatants were then incubated with each first antibody as indicated: NRS, normal rabbit serum antibody; Tyr, tyrosinase antibody; CN, calnexin antibody. The immune complexes were resuspended in buffer and heated, and the collected supernatants were aliquoted into fractions. One fraction was used for a single immunoprecipitation (−), while the remainder was subjected to a second immunoprecipitation with the noted antibody. Samples were separated on SDS/PAGE gels and immunoprecipitated bands were visualized by fluorography, as detailed in the Experimental section.

tyrosinase complexed to calnexin was confirmed by sequential immunoprecipitation with the tyrosinase antibody of the material bound to the calnexin antibody (see Figure 4, shown at the bottom of each panel). Binding of wild-type tyrosinase with calnexin was transient, the majority of wild-type tyrosinase being released from calnexin between 2 and 4 h even though the stability of tyrosinase was quite high, and significant amounts were still detectable at 16 h. In contrast, the association of calnexin with mutant tyrosinases showed different kinetics that closely paralleled their degradation. The missense mutant tyrosinases (A206T and P406L) also formed calnexin–tyrosinase complexes, but these calnexin–mutant-tyrosinase complexes remained stable for up to 8 h. Interestingly, the rates of degradation of those mutant tyrosinases were almost identical to their rates of disappearance from the calnexin complex. These results show that all mutant tyrosinases, regardless of the severity of their predicted structural abnormalities, are degraded at indistinguishable rates and that their degradation is related closely to their dissociation from calnexin.

Tyrosinase degradation can be inhibited selectively by proteasome inhibitors

As the lifespans of mutant tyrosinases were significantly shorter than that of normal tyrosinase, the degradation mechanism(s) involved are of great interest. When newly synthesized proteins fail to obtain their correct configuration, they are trapped in the ER and are consequently proteolytically degraded by the ubiquinone-proteasome pathway [18,19,33]. Two lines of evidence suggest that the degradation of wild-type tyrosinase is proteasome-dependent: (i) melanoma cells often express antigenic tyrosinase peptides on their cell surface, and proteasomes play an important role in the processing of those histocompatibility leucocyte antigen (HLA)-associated epitopes [12]; and (ii) tyrosinase retained in the ER is degraded in proteasomes in amelanotic melanoma cells [34] and in transfected cells [24]. Accordingly, to examine to what extent specific proteases are involved in the degradation of wild-type and/or mutant tyrosinases we carried out pulse–chase experiments in the presence of inhibitors of cysteine and serine proteases (M64 and

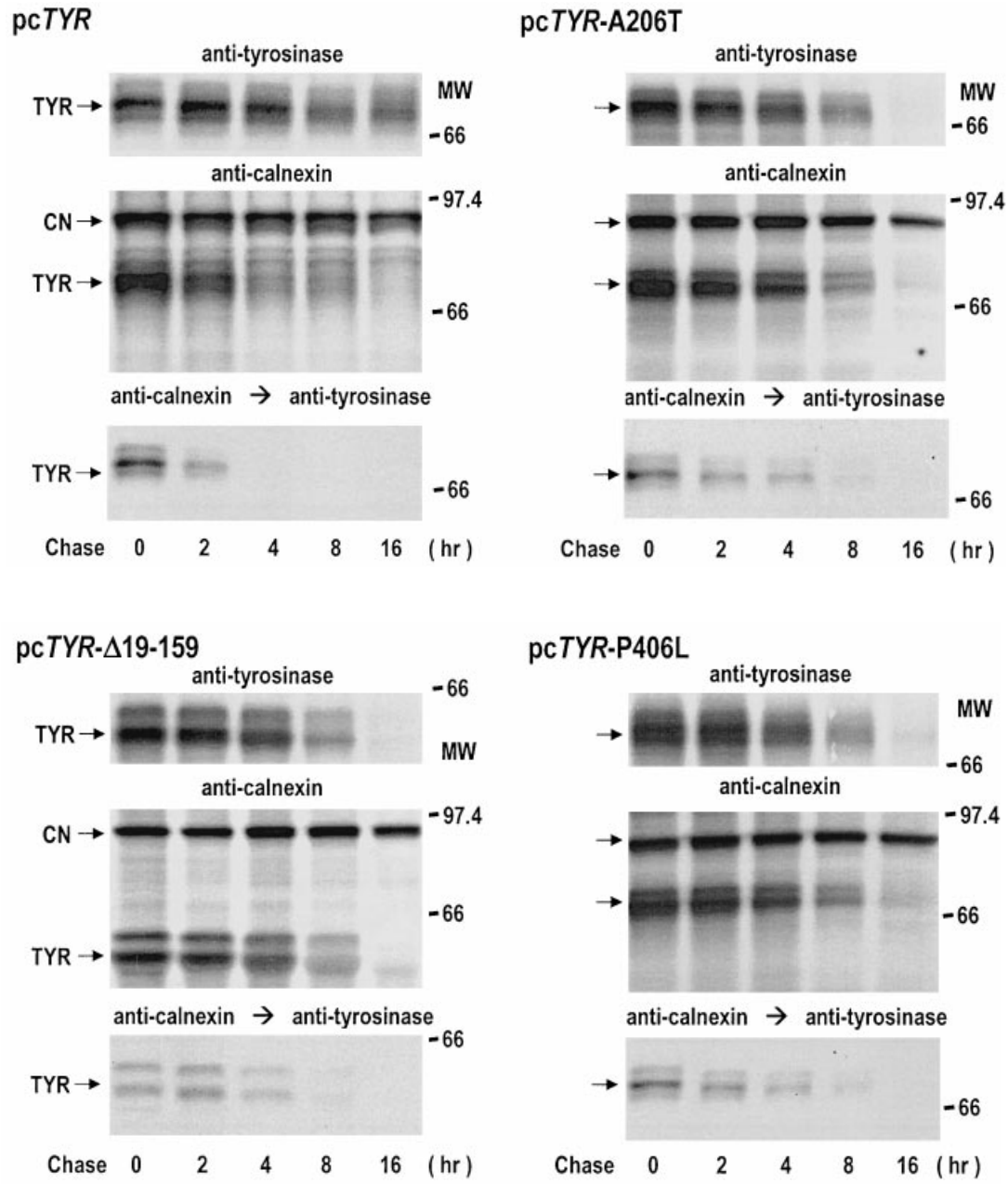

Figure 4 Degradation of mutant and wild-type tyrosinases

Transiently transfected COS7 cells were cultured for 2 days at 37 °C, and then labelled with [^{35}S]methionine for 20 min and chased for specific periods (0, 2, 4, 8 and 16 h) in medium supplemented with unlabelled methionine. The cells were then harvested and solubilized, and immunoprecipitation was performed using the tyrosinase antibody (upper part of each panel) or the calnexin antibody (middle part of each panel). For sequential immunoprecipitation, the calnexin-bound immune complexes were dissociated with heat and were subjected to a second immunoprecipitation with the tyrosinase antibody (bottom). Immune complexes were separated by SDS/PAGE and immunoprecipitated bands were visualized by fluorography. CN, calnexin band; TYR, tyrosinase band.

PMSF, respectively) and two specific proteasome inhibitors (MG132 and lactacystin) [35,36]. After labelling with [^{35}S]-methionine, COS7 cells expressing wild-type tyrosinase were chased for 24 h, whereas cells expressing mutant tyrosinases were chased for only 12 h due to the differences in their rates of degradation, as shown in Figure 5. The proteasome inhibitors, MG132 and lactacystin, significantly inhibited the degradation of wild-type and mutant tyrosinases, whereas M64 or PMSF (results not shown) did not inhibit degradation. These findings demonstrate that mutant tyrosinases, and to some extent even wild-type tyrosinase, expressed in transfected COS7 cells are primarily degraded in proteasomes. As wild-type tyrosinase is sorted to lysosomes in these cells, proteasomes are not the only place for its degradation. However, much of the wild-type tyrosinase that is over-expressed in COS7 cells transfected with pcTYR is retained in the ER (as shown above), probably since the level of endogenous calnexin in those cells is insufficient to bind and process all of it. Indeed, we showed previously that co-transfection of calnexin in COS7 cells enhanced properly sorted active tyrosinase [21].

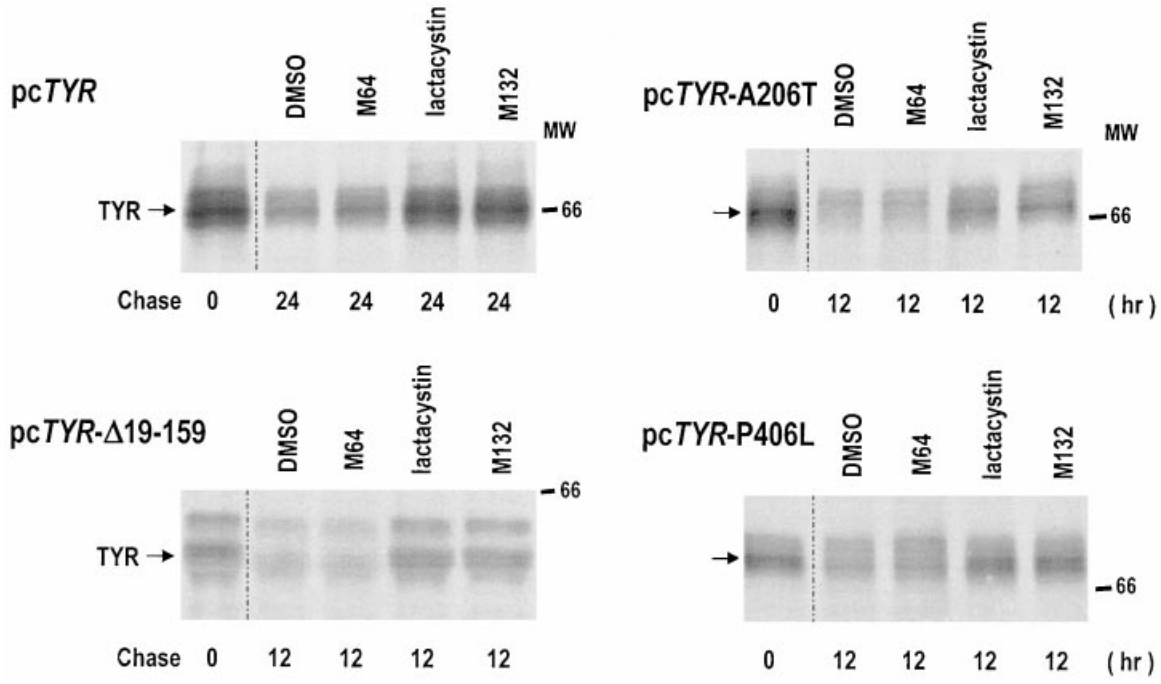

Figure 5 Inhibition of tyrosinase degradation by proteasome inhibitors

COS7 cells were transiently transfected with tyrosinase plasmids as noted and were cultured for 2 days at 37 °C. Cells were then incubated for 6 h in DMEM containing 0.1% DMSO with or without the protein inhibitors M64, MG132 or lactacystin. The cells were labelled with [^{35}S]methionine for 20 min and were then chased for 24 h (wild-type tyrosinase) or 12 h (mutant tyrosinases). Each proteinase inhibitor was added to the culture medium during the radiolabelling and chase periods. The cells were harvested and solubilized, and then immunoprecipitations were performed using the tyrosinase antibody. The collected immune complexes were separated by SDS/PAGE and immunoprecipitated bands were visualized by fluorography. TYR, tyrosinase band.

The effect of temperature on maturation and degradation of tyrosinases

From the sum of these results, the effect of temperature on the proper sorting of wild-type and mutant tyrosinases became of greater interest. To analyse further the maturation process, we transfected wild-type pcTYR or the temperature-sensitive pcTYR-P406L into COS7 cells. These cells were cultured at 37 °C or at 31 °C and were then labelled briefly with [^{35}S]-methionine, and chased for up to 20 h at the same temperature. In cells cultured at 37 °C, wild-type tyrosinase remained sensitive to endo H and only a small amount was converted into the endo H-resistant form (Figure 6). In contrast, in cells cultured at 31 °C, even though the majority of wild-type tyrosinase remained sensitive to endo H, significantly more tyrosinase was endo H-resistant. The rate of degradation of wild-type tyrosinase was visibly slower when the cells were incubated at 31 °C compared with when they were incubated at 37 °C. Both the endo H-sensitive and the endo H-resistant forms of wild-type tyrosinase had prolonged stability when cultured at 31 °C. Further, the interaction of calnexin with wild-type tyrosinase was also prolonged at 31 °C compared with its interaction at 37 °C.

The kinetics of processing of the P406L mutant tyrosinase were somewhat different from those of wild-type tyrosinase. P406L mutant tyrosinase was completely sensitive to endo H at all times during the chase when the cells were cultured at 37 °C; however, when the cells were cultured at 31 °C, P406L tyrosinase became partially resistant to endo H. In contrast to wild-type tyrosinase, the rate of disappearance of P406L tyrosinase, and its interaction with calnexin, did not significantly differ when cells were cultured at 31 °C or 37 °C. When the cells were incubated at 31 °C, even the endo H-resistant form of P406L tyrosinase, some of which exists in lysosomes (see Figure 1), was degraded quickly. In contrast, the rate of degradation of endo H-resistant or -sensitive forms of wild-type tyrosinase decreased at 31 °C. These data suggest that the endo H-resistant form of P406L tyrosinase was not processed as efficiently as wild-type tyrosinase, an observation consistent with our previous studies showing that temperature-sensitive tyrosinases had less catalytic activity than the wild-type [6,9].

DISCUSSION

OCA1 is associated with mutations of the *TYR* gene that greatly reduce or abolish tyrosinase catalytic activity and melanin production [1,2]. In this study, we have analysed the early maturation process of various OCA1 mutant tyrosinases from patients with OCA1A or OCA1B. Using immunohistochemistry, we found that all mutant tyrosinases are confined to the ER at 37 °C, whereas pulse–chase methods were used to demonstrate that all mutant tyrosinases are rapidly degraded following dissociation from calnexin, and that this degradation can be inhibited selectively by proteasome inhibitors. Sensitivity to endo H digestion showed clearly that none of the mutant tyrosinases were processed through the ER to the Golgi, as was the wild-type enzyme. Based on the sum of these observations, we conclude that OCA1 is tightly correlated with the quality control of tyrosinase processing in the ER, and does not result solely from catalytic malfunction of mutant tyrosinases in melanosomes, as was assumed previously.

COS7 and HeLa cells have often been used as recipient cells for tyrosinase transfection, and the relevance of processing in these cells versus in melanocytes is an interesting consideration.

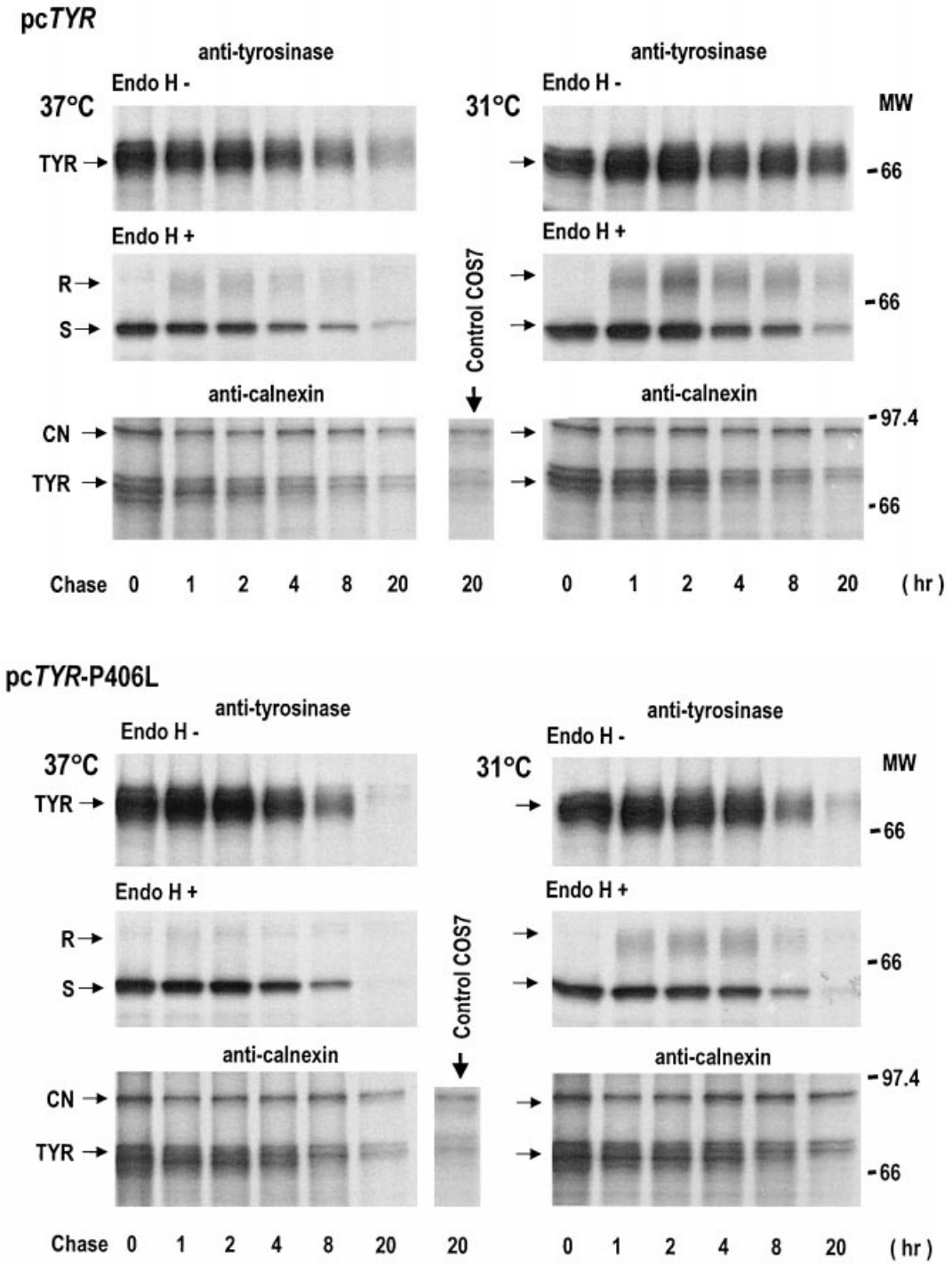

Figure 6 Effect of temperature on maturation and degradation

COS7 cells transiently transfected with pcTYR (top panel) or pcTYR-P406L (bottom panel) were cultured for 2 days at 31 °C or 37 °C. The cells were radiolabelled with [^{35}S]methionine for 10 min and were then chased for specific periods (0, 1, 2, 4, 8 and 20 h) in medium supplemented with unlabelled methionine at 31 °C or 37 °C. Cells were harvested and then solubilized, and immunoprecipitation was performed using the tyrosinase or calnexin antibodies. Immune complexes precipitated with the tyrosinase antibody were divided into two fractions, and were incubated with (+) or without (−) endo H. All samples were separated by SDS/PAGE and immunoprecipitated bands were visualized by fluorography. The panel labelled as 'Control COS7' shows the background bands immunoprecipitated with the calnexin antibody in COS7 cells transfected with pcDNA3 (mock) and chased for 20 h at 37 °C. R, endo H-resistant band; S, endo H-sensitive band; CN, calnexin band; TYR, tyrosinase band.

ER processing of wild-type tyrosinase and the R402Q temperature-sensitive mutants was recently reported using human melanocytes [23] and transfected HeLa cells [22], with results that were highly compatible with those of the current study. The rate of tyrosinase synthesis in melanocytes is much lower than in transfected cells (approx. 5-fold lower in our assays), which makes its processing through the ER more efficient in melanocytes (i.e. excess expression of tyrosinase in transfected

cells overwhelms endogenous calnexin). However, well-characterized interactions among melanosomal proteins, most notably tyrosinase, TRP1/Tyrp1 and TRP2/Dct [37,38], might influence post-translational events, complicating analysis of melanogenic enzyme processing in melanocytes. Since the folding machineries (and processes themselves) in the ER are highly conserved in mammalian cells, the folding of mutant tyrosinases in COS7 cells should accurately reflect the process that occurs in normal melanocytes. Thus transfection of non-melanocytic cells provides the opportunity to study mutants not available in cultured melanocytes and avoids interactions with other melanogenic proteins, which can influence processing (K. Toyofuku, I. Wada, J. C. Valencia, V. J. Ferrans and V. J. Hearing, unpublished work). Our results thus illustrate the critical nature of ER processing and retention as a mechanism for quality control of tyrosinase function.

The two copper-binding sites of tyrosinase, CuA and CuB, each bind one copper ion (Cu^{2+}) via co-ordination of three histidine residues. We showed previously that substitutions at any one of these histidine residues prevents copper binding and abolishes tyrosinase activity [9]. We also found in that study that copper binding at either site facilitates copper binding to the other site, and we therefore suggested that copper binding is required for the proper folding of tyrosinase. Hydrophobicity analyses of the human tyrosinase sequence indicates that highly hydrophobic clusters occur at codons 171–224 and 385–452, which correspond to the CuA and CuB regions, respectively. As these copper-binding domains contain many hydrophobic amino acids, some substitutions in those regions might deform the secondary and/or tertiary structure of the copper-binding domain. Indeed, a number of OCA1 mutations in the CuB region result in temperature-sensitive tyrosinase polypeptides [6]. Two of these substitute Glu, a negatively charged amino acid, for Arg, a positively charged amino acid, at codons 402 or 422, and deformation of secondary structure by those substitutions might prevent the mutant tyrosinases from binding copper, thereby altering its tertiary structure. The P406L mutant tyrosinase can bind copper but has little or no catalytic function [6,9]; our current results indicate that this is probably due to trapping of P406L tyrosinase by calnexin in the ER.

The folding of glycoproteins in the ER is tightly regulated by a set of abundant resident proteins including calnexin [12–14]. Tyrosinase is a type I membrane glycoprotein that has six potential glycosylation sites, and glycosylated N-linked oligosaccharides are essential for tyrosinase to obtain its proper folding and enzyme activity [39]. It was reported recently that mutations at glycosylation sites affect the expression of tyrosinase and that calnexin–tyrosinase interactions are necessary for the proper folding of tyrosinase [21] and for its catalytic function [26,27]. When the process cannot be completed, either due to the presence of aberrant amino acid(s) or to the lack of essential amino acid(s), calnexin and its substrates continue to interact by reglucosylation of deglucosylated N-linked oligosaccharides via the action of UDP-glucose:(glycoprotein) glucosyltransferase [27,40]. Misfolded proteins are degraded before reaching the Golgi apparatus, and this process may require retrotranslocation through the Sec61 channel followed by proteasomal degradation [40,41]. How prolonged association of misfolded proteins with calnexin relates to such degradation is controversial, and there are two conflicting hypotheses: (i) calnexin complexes might be pulled directly from the ER by proteasomes, indicating that the association may play a positive role in protein degradation [42] or, (ii) interactions with lectin-like chaperones might prevent degradation of unfolded or misfolded proteins [43,44]. Although we did not specifically examine the effects of abrogating calnexin interactions in this study, the first hypothesis would not be consistent with our results since degradation of tyrosinase was observed only after prolonged interaction with calnexin, and the rate of degradation was reduced for wild-type tyrosinase at a lower temperature at the same time that its association with calnexin was prolonged. Therefore, we think that it is likely that degradation of mutant tyrosinase stops when it is in a complex with calnexin and resumes when it is released.

The rates of dissociation from calnexin and degradation for all types of mutant tyrosinase studied were very similar, although the deformities in their predicted secondary and tertiary structures ranged from minimal to large. Calnexin is obviously associated with their retention and degradation in the ER, but this phenomenon suggests that another mechanism or an additional factor(s) might be involved in the detection and degradation of mutant tyrosinases. One such possibility would include a lectin that also recognizes unfolded/misfolded proteins, as does UDP-glucose:(glycoprotein) glucosyltransferase [45]. Other possibilities would include Kar2P, a homologue of Bip, which transiently binds wild-type carboxypeptidase Y and stably binds mutant carboxypeptidase Y in yeast [46], or Hsp 70, which participates in the degradation of misfolded proteins [42]. In yeast, unfolded proteins having $Man_8GluNAc_2$ are vulnerable to degradation, whereas correctly folded proteins possessing the same oligosaccharides are resistant. Interestingly, oligosaccharides attached to correctly folded proteins are subsequently trimmed to stable forms having $Man_{7-5}GluNAc_2$ [47]. The mannose-trimming process is relatively slow and is a key point after which misfolded proteins are degraded while correctly folded proteins advance to the next step for further sorting. A putative Man_8 lectin has been proposed as the receptor of terminally misfolded glycoproteins, which may link misfolding to retrotranslocation followed by proteasomal degradation. A similar mechanism appears to occur in eukaryotes [48]; thus our results may reflect the principles of quality control in the ER, mannose trimming of tyrosinase mutants with varying degrees of deformities proceeding at equal rates and mutant proteins being transferred from calnexin to the putative lectin when nascent glycoproteins are retained in the ER for longer than a certain period.

In summary, this study has shown that a wide range of mutant tyrosinases are retained by calnexin in the ER and are degraded in proteasomes rather than being further processed and sorted. Among the substitution mutations of *TYR* examined, A206T and P406L presumably have the least potential to change the secondary or tertiary structure of tyrosinase. A206T is a mild substitution of neutral amino acids in the CuA region, and although the P406L mutation is less severe and occurs in the CuB region, the mutant enzyme retains the ability to bind copper. Therefore, it is interesting to note that even these minimally mutant tyrosinases are trapped by calnexin and do not exit from the ER to the same extent as the more severely mutant enzymes such as the deletion mutants. It seems likely that similar phenomena occur in other mutant tyrosinases found in OCA1A and OCA1B, since calnexin recognizes mutant forms of tyrosinase in such a sensitive and specific manner. This new understanding of the vigorous quality control of tyrosinase processing in melanocytes provides a new basis for understanding the aetiology of OCA, and perhaps of other hypopigmentary conditions.

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