Regulation of Tyrosinase Processing and Trafficking by Organellar pH and by Proteasome Activity*

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Pigmentation of the hair, skin, and eyes of mammals results from a number of melanocyte-specific proteins that are required for the biosynthesis of melanin. Those proteins comprise the structural and enzymatic components of melanosomes, the membrane-bound organelles in which melanin is synthesized and deposited. Tyrosinase (TYR) is absolutely required for melanogenesis, but other melanosomal proteins, such as TYRP1, DCT, and gp100, also play important roles in regulating mammalian pigmentation. However, pigmentation does not always correlate with the expression of TYR mRNA/protein, and thus its function is also regulated at the post-translational level. Thus, TYR does not necessarily exist in a catalytically active state, and its post-translational activation could be an important control point for regulating melanin synthesis. In this study, we used a multidisciplinary approach to examine the processing and sorting of TYR through the endoplasmic reticulum (ER), Golgi apparatus, coated vesicles, endosomes and early melanosomes because those organelles hold the key to understanding the trafficking of TYR to melanosomes and thus the regulation of melanogenesis. In pigmented cells, TYR is trafficked through those organelles rapidly, but in amelanotic cells, TYR is retained within the ER and is eventually degraded by proteasomes. We now show that TYR can be released from the ER in the presence of protonophore or proton pump inhibitors which increase the pH of intracellular organelles, after which TYR is transported correctly to the Golgi, and then to melanosomes via the endosomal sorting system. The expression of TYRP1, which facilitates TYR processing in the ER, is down-regulated in the amelanotic cells; this is analogous to a hypopigmentary disease known as oculocutaneous albinism type 3 and further impairs melanin production. The sum of these results shows that organelar pH, proteasome activity, and down-regulation of TYRP1 expression all contribute to the lack of pigmentation in TYR-positive amelanotic melanoma cells.

1 The abbreviations used are: TYR, tyrosinase; ALLN, N-acetyl-Leu-Leu-Leu-norleucine; Baf, bafilomycin A1; CCM, concanamycin A; CHX, cycloheximide; ER, endoplasmic reticulum; DCT, tyrosinase-related protein 2; Endo H, endoglycosidase H; MG132, benzyloxycarbonyl-Leu-Leu-leucinal; MITF, microphthalmia-associated transcription factor; Mna, monensin; OCA, ocularcutaneous albinism; PNGase F; PeptideN-glycosidase F; TGN, trans-Golgi network; TYRP1, tyrosinase-related protein 1; DAPI, 4,6-diamidino-2-phenylindole; MES, 4-morpholinethanesulfonic acid; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; vATPase, vacuolar-type proton ATPase; mAb, monoclonal antibody; AP, adaptor protein; LAMP, lysosome-associated membrane protein; PBS, phosphate-buffered saline.
the target organelle, the melanosome, by a vesicular transport system (28–30). Interestingly, TYRP1 and DCT, two proteins closely related to TYR, undergo similar processing and sorting, although the sorting vesicle system used to deliver them from the TGN to melanosomes is presumably unique for each of those proteins (31, 32). Even gp100 is processed through the ER, but is transferred to early melanosomes directly from the ER or from the early Golgi (33) in a manner distinct from the TYR-related proteins.

The processing of TYR in the ER requires the presence of the chaperone calnexin, which increases the ER retention time for TYR necessary for its binding of copper and its conformational folding (34–36). The most common mutations of TYR result in oculocutaneous albinism type 1 (OCA1), which is associated with the ER retention of the protein, presumably as the result of enzyme misfolding (27, 37, 38); the misfolded TYR is then degraded by proteasomes. In OCA3, normal TYR is produced, but mutations in TYRP1 result in the retention of the wild-type TYR in the ER and its proteolysis by proteasomes; TYRP1 seems to act as a chaperone for TYR in the ER (27). Thus the reduction in TYR function in amelanotic melanoma cells could be mediated by the quality control system of the ER, because selective retention in the ER and subsequent degradation by proteasomes occurs in several genetic diseases (39–41). The sum of these studies underscore the fact that proper folding and processing of TYR in the ER is crucial for its enzymatic activity, delivery to melanosomes, and subsequent melanin synthesis. Thus, disruption of melanin synthesis may result not only from mutations at the TYR locus but also from mutations at a number of other loci involved in TYR processing and transport.

Melanosomes are known to be acidic organelles that when mature can have a pH as low as 4.0 (42, 43). It has been assumed that this low melanosomal pH facilitates melanogenesis (44–46). Recently, the activation of melanogenesis by selective vacuolar type proton pump inhibitors, bafloymycin A1 (Baf) and concanamycin A (CCM), was shown in amelanotic human melanoma cells and in mouse melanoma cells, which express TYR but do not produce pigment (47). Further, Baf and CCM induce melanin synthesis in pink-eyed dilution gene (p)-null melanocytes by affecting early TYR processing and trafficking rather than by simply affecting activity at the melanosomal level (48, 49). Fuller et al. (50) showed that melanocytes derived from caucasian donors respond to those agents by producing melanin after activation of TYR, whereas melanocytes derived from black donors were refractory to those agents, suggesting that intracellular pH might regulate constitutive skin color. Baf and CCM, macrolide antibiotics that at low concentrations specifically inhibit vacuolar-type proton ATPases (vATPase) (51), have been commonly used to neutralize acidic compartments such as endosomes, in which the low luminal pH is known to affect receptor-ligand interactions and protein sorting (49, 52, 53). Recent studies have shown that Baf inhibits the delivery of endocytosed material from endosomes to lysosomes (54–56). Baf was shown to function by inhibiting the formation of carrier vesicles operating between early and late endosomes (54) and to block the association of a subset of coatamer subunits with early endosomal membranes (57, 58).

In this study, we examined the expression, processing, and trafficking mechanisms of various melanosomal proteins (TYR, TYRP1, DCT, gp100, and MART1) in amelanotic and in melanotic melanoma cells using immunological, biochemical, and molecular approaches to characterize melanosomal localization in early melanosomes, endosomes, coated vesicles, Golgi apparatus, and ER. Those organelles hold the key to the understanding of biogenesis and pigment synthesis of melanosomes and how that might be disrupted in unpigmented melanocytes that express normal levels of melanogenic proteins. We confirm that the processing of TYR is altered in amelanotic melanoma cells, and we show that its retention in the ER can be corrected in the presence of protonophore or proton pump inhibitors, which increase the pH of intracellular organelles, after which TYR is transported correctly from the ER to the Golgi, further glycosylated, and then sorted to melanosomes. The sum of these results shows that organelar pH, proteasome activity, and the down-regulation of TYRP1 expression may all contribute to the lack of pigmentation in TYR-positive amelanotic melanoma cells.

MATERIALS AND METHODS

Cell Cultures—SK-MEL-28 amelanotic human melanoma cells were obtained from the ATCC (Manassas, VA) and cultured in minimum essential medium (Invitrogen) containing 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2.8 μg/ml sodium bicarbonate, 2 mM l-glutamine, and penicillin/streptomycin. M1NT-pigmented human melanoma cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) as described previously (33).

Antibodies and Reagents—αPEP1h, αPEP7h, αPEP8h, and αPEP13h antibodies were generated in rabbits against synthetic peptides corresponding to the carboxyl termini of human TYRP1, TYR, DCT, and gp100, respectively, as described previously (59–62). Other antibodies to melanosomal proteins used in this study included M2–9E3, a mouse mAb against human MART-1 (Neomarkers, Fremont, CA), HMB45, a mouse mAb against human gp100 (Dako, Carpinteria, CA), and T311, and a mouse mAb against human TYR (Novacastro Laboratories, Newcastle upon Tyne, UK). We used other antibodies to check for localization in subcellular organelles as follows: CD107a, a mAb against the ER, Golgi apparatus (48), early endosomes, late endosomes (63), adaptor protein complex-3 (AP-3), and adaptor protein complex-1 (AP-1), respectively (all from BD Transduction Laboratories, Lexington, KY).

Proteasome inhibitors N-Acetyl-Leu-Leu-norleucinal (ALLN) and benzyloxycarbonyl-Leu-Leu-leucinal (MG132) were purchased from Calbiochem. Cycloheximide (CHX, an inhibitor of protein synthesis), the vATPase inhibitors (Baf and CCM), and the proteophore monensin (Mon) were purchased from Sigma. All inhibitors were dissolved in dimethyl sulfoxide.

Cellular Fractionation—For purification of melanosomes, we used the protocol originally described in Ref. 33 with modifications. Briefly, confluent monolayers of SK-MEL-28 cells were harvested with 0.05% trypsin, 0.53 mM EDTA (Invitrogen) and washed once in 0.25 M sucrose, MES buffer (0.1 M sodium MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl2, 2 mM sodium bicarbonate, 2 mM l-glutamine, and penicillin/streptomycin) and centrifuged at 1,250 g for 5 min at 4 °C. Specimens were then homogenized on ice and various fractions were analyzed by Western blotting using antibodies as indicated in the figure legends.

We used a standard technique (64) to purify endosomes and coated vesicles. Briefly, SK-MEL-28 cells were harvested and washed once in MES buffer (0.1 M sodium MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl2, 0.02% sodium azide, and a protease inhibitor mixture) by centrifugation at 1,250 × g for 5 min at 4 °C. Specimens were then homogenized on ice using 20 strokes of a Dounce glass/glass homogenizer and centrifuged at 10,000 × g for 30 min at 4 °C. The pellet was resuspended in 2.0 M sucrose and layered at the bottom of a 1.0–2.0 M sucrose step (1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M) gradient. The gradient was centrifuged at 100,000 × g in a Beckman SW 28 swinging-bucket rotor for 1 h at 4 °C, and the various layers of the fraction were carefully recovered. The 1.0 M sample was then layered in the middle of a 0.8–1.4 M sucrose step (0.8, 1.0, 1.2, and 1.4 M) gradient. That gradient was again centrifuged at 100,000 × g for 1 h at 4 °C and the 0.8 and 1.0 M fractions were carefully recovered. The various layers of fractions were analyzed by Western blotting using antibodies as indicated in the figure legends.

We used a standard technique (64) to purify endosomes and coated vesicles. Briefly, SK-MEL-28 cells were harvested and washed once in MES buffer (0.1 M sodium MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl2, 0.02% sodium azide, and a protease inhibitor mixture) by centrifugation at 1,250 × g for 5 min at 4 °C. Specimens were then homogenized on ice using 20 strokes of a glass/glass homogenizer and centrifuged at 19,000 × g for 40 min at 4 °C. The supernatant was recovered and further centrifuged at 19,000 × g for 30 min at 4 °C. That pellet was again homogenized on ice using 10 strokes of a glass:glass homogenizer in Percoll/sucrose-MES buffer (12.5% Percoll, 12.5% sucrose, MES) and centrifuged at 35,000 × g for 40 min at 4 °C. The pellet was recovered, resuspended in buffer, and used as the endosome-rich fraction. The supernatant was recovered and was again centrifuged...
at 82,000 × g for 70 min at 4 °C, the resulting pellet was resuspended in buffer and used as the coated vesicle fraction. For purification of ER and Golgi apparatus, we used a previously published method (65). Briefly, SK-MEL-28 cells were harvested and washed once in 0.25 M sucrose by centrifugation at 1,000 × g for 5 min at 4 °C. Specimens were then homogenized on ice using 20 strokes of a glass-grass homogenizer and centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was recovered and centrifuged at 20,000 × g for 30 min at 4 °C. That supernatant was recovered and was further centrifuged at 105,000 × g in a Sorvall T1270 rotor for 60 min at 4 °C. The pellet was resuspended in 1.35 M sucrose and layered in the middle of a 0.8, 1.0, 1.2, 1.35, and 2.1 M sucrose step (0.8, 1.0, 1.2, 1.35, and 2.1 M gradient), which was then centrifuged at 100,000 × g in a Beckman SW 28 swinging-bucket rotor for 6 h. The 0.8 and 1.0 M layers were carefully recovered and used as the Golgi-rich fraction, whereas the layers of the 1.35 and 2.1 M fractions were recovered and used as the ER-rich fraction.

Reverse Transcription-PCR—Total RNA was extracted from cells using a RNA isolation kit (Qiagen, Hilden, Germany) as described by the manufacturer. cDNA was prepared from 10 μg of total RNA using Moloney leukemia virus reverse transcriptase (MMLV-RT, Invitrogen). PCR reactions consisted of 30 cycles for TYR, TYRP1, and G3PDH. PCR reactions were initiated with a “cold start” followed by denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and polymerization at 72 °C for 60 s. The primer sequences used were as follows: TYR sense primer 5′-TTGGCACATTCTTGATCCG-3′; TYRP1 antisense primer 5′-GCCAGTCTGCATGCTGCTT-3′; TYRP1 antisense primer 5′-AGGAGGTCTTCCAGCTTTG-3′; G3PDH sense primer 5′-ACCACAGGCTCATGATC-3′; G3PDH antisense primer 5′-TCCACACCCCTTGTC-3′. PCR products for TYR, TYRP1, and G3PDH were 284, 500, and 458 bp, respectively, and were resolved in 1.5% agarose gels containing ethidium bromide in parallel with DNA molecular weight markers. Blots were quantitated using ScionImage software (Scion, Frederick, MD).

Electron Microscopy—Electron microscopy was performed as reported previously (33). Briefly, cells were harvested and collected by centrifugation at 4 °C. After several washes in PBS, cells were fixed overnight at 4 °C in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The samples were then stored in PBS for 6 h at 4 °C. After three washes in PBS, the cells were fixed in 4% paraformaldehyde and 0.1 M glycine in 0.1 M sodium cacodylate buffer (pH 7.3). The samples were then stored in PBS for 1 h at room temperature. After three washes with PBS-T, the cells were permeabilized with 0.01% Triton X-100 for 3 min at room temperature and then incubated with primary antibodies diluted (as noted in the figure legends) in 5% normal goat serum and 5% normal horse serum for 1 h at room temperature. The cells were then be degraded by proteasomes as occurs in OCA3 melanocytes (27). The latter was indicative of colocalization of the red and green fluorescence signals. All preparations were stained by immunofluorescence microscopy. We used Western blotting to examine the expression of melanosomal proteins in amelanotic SK-MEL-28 cells and in pigmented MNT-1 melanoma cells. As shown in Fig. 1A, MNT-1 cells were positive for all five known melanosomal proteins (TYR, TYRP1, DCT, G3PDH, and PEPT1h) as described previously (27). Cells were incubated with Met/Cys-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed fetal bovine serum. For pulse-chase experiments, cells were pulsed and chased for specific periods at 37 °C (as detailed in the figure legends) in minimum Eagle’s medium containing 1.0% unlabeled methionine supplemented with or without 10 μM Mon or 25 μM Baf. Cells were harvested and solubilized overnight at 4 °C in immunoprecipitation lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.01% SDS, pH 7.4, containing complete protease inhibitor mixture). Cell extracts were incubated with 40 μl of normal rabbit serum for 2 h at 4 °C with continuous mixing and were then incubated with 150 μl of protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 2 h at 4 °C with continuous mixing. For immunoprecipitation, the supernatants were collected by centrifugation at 4 °C and incubated with 10 μl of aPEPTh or normal rabbit serum as a control for 2 h at 4 °C with continuous mixing. The immunocomplexes were separated by incubation with 20 μl of protein G-Sepharose 4 Fast Flow for 2 h at 4 °C with continuous mixing and were further washed six times with immunoprecipitation lysis buffer. The final pellets were mixed with Tris-glycine SDS sample buffer (2×) supplemented with 5% 2-mercaptoethanol and boiled for 5 min. Samples were separated on 6–16% SDS-PAGE, and the separated protein bands were visualized by fluorography using Enlightening (PerkinElmer Life Sciences).

RESULTS

Expression of Melanosomal Proteins and the Ultrastructure of SK-MEL-28 and MNT-1 Melanoma Cells—We used Western blotting to examine the expression of melanosomal proteins in amelanotic SK-MEL-28 cells and in pigmented MNT-1 melanoma cells. As shown in Fig. 1A, MNT-1 cells were positive for all five known melanosomal proteins (TYR, TYRP1, DCT, gp100, and MART-1). SK-MEL-28 cells were positive for TYR, gp100, DCT, and MART-1, but there was had only a barely detectable band of TYRP1. Reverse transcription-PCR analysis of those melanosogenic genes correlated with the protein expression patterns and confirmed that TYRP1 is transcribed at very low levels in SK-MEL-28 cells (Fig. 1B). Quantitation of the bands after normalization against G3PDH revealed that the TYR mRNA level in SK-MEL-28 cells is about 40% that detected in MNT-1 cells, whereas the level of TYRP1 mRNA in SK-MEL-28 cells is about 10% that detected in MNT-1 cells.

Kobayashi et al. (67) showed that TYRP1 plays an important role in stabilizing TYR, and mutations affecting TYRP1 function have been shown to result in hypopigmentation such as found in OCA3 (27, 68). The mobility patterns in the Western blots shows that TYR in SK-MEL-28 cells appears as the immature 60-kDa form rather than the fully glycosylated 70–85 kDa form detected in MNT-1 cells, suggesting that TYR may then be degraded by proteasomes as occurs in OCA3 melanocytes. Consistent with this expectation, TYR was consistently less abundant in SK-MEL-28 cells compared with MNT-1 cells. These results demonstrate clearly that TYR in amelanotic SK-
MEL-28 melanoma cells is not glycosylated correctly compared with pigmented MNT-1 melanoma cells. The ultrastructure of these cells is shown in Fig. 1C. Only stages I and II (unmelanized) early melanosomes were seen in the amelanotic SK-MEL-28 melanoma cells with none in more advanced stages, whereas MNT-1 cells also contained melanized stage III and IV melanosomes in addition to those early stage melanosomes.

Subcellular Distribution of TYR in SK-MEL-28 cells and MNT-1 Cells—To investigate the subcellular localization of TYR in SK-MEL-28 cells and in MNT-1 cells, we used immunohistochemical staining with αPEP7h (which is specific for TYR) to compare its distribution in the ER (using Bip/GRP78 as a marker), the Golgi (using Vti1b), early endosomes (using EEA1), late endosomes (using syntaxin 8), stage I melanosomes (using αPEP13h), and stage II melanosomes (using HMB45). In the merged images shown in Fig. 2, yellow indicates colocalization of the two signals. As shown in Fig. 2A, TYR in the pigmented MNT-1 melanoma cells colocalized with markers for the ER, Golgi, early and late endosomes and early (stage I and II) melanosomes.

In contrast, in SK-MEL-28 cells (Fig. 2B), the majority of MEL-28 melanoma cells is not glycosylated correctly compared with pigmented MNT-1 melanoma cells.

The ultrastructure of these cells is shown in Fig. 1C. Only stages I and II (unmelanized) early melanosomes were seen in the amelanotic SK-MEL-28 melanoma cells with none in more advanced stages, whereas MNT-1 cells also contained melanized stage III and IV melanosomes in addition to those early stage melanosomes.
TYR colocalized with the ER in the immediate perinuclear area, and very little TYR was found in the Golgi in early or late endosomes or in melanosomes. Thus, most of the TYR in amelanotic melanoma cells was retained in the ER, although a minor amount was detectable in stage I melanosomes. Thus, TYR trafficking to early melanosomes is dramatically reduced in amelanotic SK-MEL-28 melanoma cells, which is consistent with the lack of production of melanin therein.

**Distribution of Melanosomal Proteins in Subcellular Fractions of SK-MEL-28 Cells**—We used Western blotting to examine the distribution of melanosomal proteins in purified subcellular fractions of pigmented MNT-1 cells (not shown) and amelanotic SK-MEL-28 cells (Fig. 3). Early melanosomes can be separated efficiently by differential and sucrose density gradient centrifugation as detailed under “Materials and Methods.” The ER, Golgi, endosome, and coated vesicle fractions were purified by the fractionation techniques detailed under “Materials and Methods.” Full-length gp100 (100 kDa, as detected by αPEP13h) was distributed in the ER and Golgi fractions and in the 0.8 and 1.0 M sucrose fractions, whereas processed gp100 (~35 kDa, as detected by HMB45) was found in the 1.4 to 1.8 M sucrose fractions (data not shown). Note that gp100 was not present in the coated vesicle or endosomal fractions (contrast with TYR and MART-1 as discussed below).
We have recently demonstrated (33) that stage I melanosomes are recognized by aPEP13h and that stage II melanosomes are recognized by HMB45 following the proteolytic cleavage of gp100. From these reactivity patterns, it is clear that the 0.8 M sucrose fraction contained only stage I melanosomes, that the 1.0 M and 1.2 M sucrose fractions contained stage I and II melanosomes, and that the 1.4–1.8 M sucrose fractions contained stage II melanosomes; this is consistent with the contents of those fractions as previously demonstrated by electron microscopy (33).

TYR and MART-1 were detected in the ER, Golgi, coated vesicle, and endosome fractions and in stage I melanosomes of...
SK-MEL-28 cells. This reactivity pattern is identical to that recently reported for mouse melanoma cells (32). In contrast, DCT was distributed in the ER and Golgi but was not present in stage I melanosomes. TYRP1 was not detectable in any fractions (not shown), which is consistent with the results presented above. Interestingly, the coated vesicle fraction (positive for AP-1 and AP-3 as expected) contained TYR and MART-1 but not DCT or gp100. These results are quite distinct from the patterns found for TYR localization in pigmented MNT-1 cells, where TYR is abundant in stage II melanosomes but is almost completely lacking in stage I melanosomes (33).

To demonstrate the relative purity of these enriched subcellular fractions, we used standard antibodies for organelle markers. As expected, the ER fraction contained the majority of Bip/GRP78 and calnexin, the Golgi fraction contained the majority of Vit1b, the endosome fraction contained the majority of EEA1, and the coated vesicle fraction contained the majority of AP-1.

**Endo H Sensitivity of TYR in SK-MEL-28 Cells and MNT-1 Cells**—To further characterize the processing of TYR in amelanotic SK-MEL-28 melanoma cells and pigmented MNT-1 cells, we assessed its sensitivity to Endo H, an enzyme that removes high mannose-type carbohydrates from N-linked glycoproteins. This conversion occurs in the medial Golgi region, and when proteins are correctly processed through the ER and Golgi, they become resistant to Endo H yet remain sensitive to PNGase F.

In MNT-1 cells, the majority of TYR was completely resistant to Endo H, showing that TYR in those pigmented melanoma cells is correctly transported to the medial Golgi region or TGN and is correctly glycosylated (Fig. 4A). In contrast, in SK-MEL-28 cells, the majority of TYR was sensitive to Endo H, although a very small amount of TYR was Endo H-resistant. These results are consistent with an earlier study (39) and show that TYR in amelanotic SK-MEL-28 melanoma cells is not glycosylated correctly and is retained in the ER, also confirming the immunohistochemical results described above.

We then examined the sensitivity of TYR to Endo H and PNGase F in the ER, Golgi, endosome, and stage I melanosome fractions purified from SK-MEL-28 cells. As shown in Fig. 4B, TYR in the ER of SK-MEL-28 cells was sensitive to Endo H, whereas TYR that reached the Golgi, endosomes, and stage I melanosomes was resistant to Endo H. These results confirm that although the majority of TYR (cf. Fig. 4A) is not glycosylated correctly in SK-MEL-28 cells, the minor amount of TYR that is processed through the Golgi, endosomes, and early melanosomes is correctly glycosylated.

**Stability and Degradation of TYR in SK-MEL-28 cells and MNT-1 Cells**—To examine the processing and stability of TYR in SK-MEL-28 cells and MNT-1 cells, cultures were pulse-chase radiolabeled with [125I]Met/Cys, and extracts of the labeled proteins were then immunoprecipitated with aPEP7h, which recognizes TYR. The synthesis and processing of TYR in
SK-MEL-28 cells and MNT-1 cells are shown in Fig. 4C. The immature form of TYR was detected as a 60-kDa band at 0 h chase in MNT-1 cells and was quickly converted from that immature form to the mature form (75 kDa) within 1.5–3 h. Most of the radiolabeled TYR in MNT-1 cells was detectable even after a 24-h chase. In contrast, SK-MEL-28 cells were much less efficient in processing TYR, and the TYR was degraded quickly (very little of the TYR synthesized was glycosylated to the 75-kDa mature form, and most of the TYR had been degraded within 3 h of chase). These results suggest that the degradation of TYR is markedly accelerated in amelanotic melanoma cells compared with pigmented melanoma cells.

We also used another independent approach to investigate the stability of TYR in SK-MEL-28 cells and MNT-1 cells, using CHX to inhibit protein synthesis and analyzing TYR levels by Western blotting. As shown in Fig. 4D, TYR levels in SK-MEL-28 cells disappeared quickly in the presence of CHX compared with the stable nature of TYR in MNT-1 cells, confirming the metabolic labeling and immunoprecipitation results described above.

**Effect of Proteasome Inhibitors on TYR in SK-MEL-28 Cells and MNT-1 Cells**—To investigate the role of proteasomes on TYR in SK-MEL-28 cells and MNT-1 cells, cells were incubated for varying times with proteasome inhibitors such as MG132 or FIG. 6. Subcellular distribution of tyrosinase after neutralization of intracellular pH. SK-MEL-28 melanoma cells were cultured in the presence of Mon, Baf, or CCM for 3 h. Cells were fixed and stained with antibodies (αPEP7h at 1:20, Bip/GRP78 at 1:10, EEA1 at 1:40, or HMB45 at 1:10). The polyclonal antibodies were reacted with goat anti-rabbit IgG labeled with Texas Red (1:100), and the monoclonal antibodies were reacted with horse anti-mouse IgG labeled with fluorescein (1:100) followed by nuclear counterstaining with DAPI. Reactivity was classified into three categories according to whether they showed green, red, or yellow fluorescence.
ALLN in the presence of CHX, and the extracted proteins were analyzed by Western blotting. TYR levels in MNT-1 cells were quite stable even after 6 h and were not changed in the presence or absence of MG132 (Fig. 4E). In contrast, TYR levels in SK-MEL-28 cells were rapidly reduced in the presence of CHX, even within 3 h, but when they were also treated with MG132 (or ALLN, not shown), that degradation was dramatically abrogated. TYR levels increased in a dose- and time-dependent manner following treatment of SK-MEL-28 cells with MG132 or ALLN (data not shown). These results demonstrate that TYR in amelanotic melanoma cells is highly sensitive to proteasome inhibitors and is therefore actively degraded by proteasomes.

**Effect on TYR of Neutralizing the pH of Acidic Intracellular Organelles**—To clarify the effects of organellar pH on TYR processing and trafficking in SK-MEL-28 cells and MNT-1 cells, cells were incubated with the protonophore Mon or with proton pump inhibitors such as Baf or CCM, and the extracted proteins were analyzed by Western blotting. TYR levels in MNT-1 cells were not altered significantly in the presence or absence of Mon or Baf (Fig. 5A). In contrast, TYR levels in SK-MEL-28 cells quickly decreased following treatment with CHX, but in the presence of Mon or Baf (or CCM, not shown) TYR levels were partially stabilized. Furthermore, in SK-MEL-28 cells, TYR accumulated in a dose- and time-dependent manner following treatment with Mon, Baf, or CCM (not shown). These results demonstrate that TYR in amelanotic SK-MEL-28 melanoma cells is highly sensitive to agents that raise the pH of intracellular organelles.

We next used metabolic labeling to investigate the stability of TYR in SK-MEL-28 cells in the presence or absence of Mon or Baf. Cells were pulse-chase radiolabeled with [35S]Met/Cys, and extracts of the labeled cultures were then immunoprecipitated with an antibody to TYR (αPEP7h). As shown in Fig. 5B, the immature 60-kDa form of TYR was detected at 0 h chase following treatment with Mon or Baf, and the mature glycosylated 75-kDa form of TYR appeared from 1.5 h to 6 h. The stability of TYR was also prolonged after treatment with Mon or Baf, suggesting that the degradation of TYR in amelanotic SK-MEL-28 melanoma cells is abrogated by neutralizing the pH of acidic intracellular organelles.

To more closely clarify the processing of TYR in SK-MEL-28 cells, we assessed its sensitivity to Endo H in response to changes in intracellular pH. Before treatment with Mon, Baf, or CCM, the majority of TYR was sensitive to Endo H (as shown above in Fig. 4A), whereas after varying times of treatment with Mon, Baf, or CCM, much of the TYR became Endo H-resistant (Fig. 5C), which demonstrates that neutralizing the intracellular pH stimulates the processing of TYR from the ER to the Golgi.

We used Western blotting to examine the distribution of TYR during the purification of early melanosomes and endosomes. Before treatment with Mon or CCM, TYR was detected only in fractions containing stage I melanosomes and in the endosome fraction (as shown in Fig. 3), whereas after treatment with Mon or CCM, TYR distribution to stage II melanosomes (1.4–1.8 M) was dramatically increased (Fig. 5D, compare with Fig. 3). Further, TYR and EEA1 content in the endosome fraction was reduced following incubation with CCM, but following treatment with Mon, the level of TYR was increased significantly in the endosome fraction. These results suggest that treatment with the protonophore Mon directs TYR to be correctly transported to stage II melanosomes via the endosome network, but that treatment with the proton pump inhibitor CCM allows TYR to be sorted to stage II melanosomes from the TGN without trafficking through the endosomal system.

**Subcellular Distribution of TYR after Neutralization of Intracellular pH**—To investigate the subcellular localization of TYR in amelanotic SK-MEL-28 melanoma cells after neutralization of organellar pH, we used immunohistochemical staining to compare its distribution in the ER, early endosomes, and stage II melanosomes (Fig. 6). TYR staining was detected by red fluorescence, whereas the others were detected by green fluorescence; in the merged images, yellow indicates the colocalization of the two signals. Before treatment with Mon, Baf, or CCM, the majority of TYR colocalized with the ER in the perinuclear area, whereas after treatment with any of those three compounds, the yellow fluorescence in the ER was reduced and the green fluorescence was increased. Therefore, TYR is not retained in the ER in the presence of Mon, Baf, or CCM and is more efficiently sorted to the Golgi. In untreated SK-MEL-28 cells, some TYR colocalized with early endosomes, whereas after treatment with Baf or CCM, the yellow fluorescence virtually disappeared, confirming the Western blotting results described above and showing that TYR is trafficked beyond the early endosomes after neutralization of intracellular pH. Similarly, prior to treatment with Mon, Baf, or CCM, a small amount of TYR colocalized with stage II melanosomes (HMB45) in the perinuclear area, whereas after treatment with any of those agents, the amount of TYR in stage II melanosomes increased dramatically. These results suggest that the processing of TYR is rescued in amelanotic melanoma cells treated with protonophore or proton pump inhibitors, showing that the processing of TYR can be corrected by adjusting the intracellular organelar pH.

**DISCUSSION**

More than 100 distinct genes play direct or indirect roles in regulating mammalian pigmentation (69). Many of those genes encode proteins that are localized in melanosomes, specialized pigment organelles produced only in melanocytes. Those gene products modulate the type and amount of melanin produced and/or its processing and distribution of melanosomes. The known melanosomal proteins are involved in melanogenesis as catalytic and/or structural components and include TYR, TYRP1, DCT, MART-1, and gp100 (60, 70). Although the processing and sorting of those proteins are not completely understood, they are known to be synthesized and translocated into the ER and eventually into the Golgi where their post-translational processing and glycosylation take place (36, 71). Following that processing, they seem to take distinct routes to traffic to melanosomes, with the majority of melanosomal proteins predominantly going to stage II melanosomes, although that distribution is disrupted in amelanotic melanoma cells. Despite their high structural similarity and conserved primary sequences, the three tyrosinase-related proteins use distinct routes to move from the TGN to early melanosomes: TYR uses the AP-3 system, TYRP1 uses the AP-1 system, and DCT uses yet another unknown sorting vesicle system (32, 41, 72). The sorting system for MART-1 is not yet known, but its distribution patterns are highly similar to TYR. gp100 is perhaps the most uniquely processed of the melanosomal proteins; after processing through the ER and Golgi, it is normally delivered to stage I melanosomes without going through the endosomal system (33, 66). Following the maturation of stage I to stage II melanosomes, which is coincident with the cleavage and refolding of gp100, the enzymatic components are delivered, and the synthesis of melanin usually ensues (2).

To investigate the subcellular distribution of melanosomal proteins in amelanotic melanoma cells, which produce melanosomal proteins and thus should be pigmented, we used immunofluorescence staining and Western blotting in conjunction with the purification of subcellular organelles. In pigmented
MNT-1 cells, the majority of TYR was processed correctly through the ER, Golgi, and endosomes and was delivered to stage II melanosomes (33). In contrast, the majority of TYR in amelanotic SK-MEL-28 melanoma cells was retained in the ER, although a small amount of TYR was glycosylated correctly and was found in the Golgi, endosomes, and early melanosomal fraction (but primarily in stage I melanosomes). The effects of this aberrant processing on disruption of melanosomal maturation and tyrosinase function are shown in this study, using confocal immunohistochemistry, electron microscopy, subcellular fractionation, and Western blotting, as well as metabolic labeling and immunoprecipitation.

We further analyzed the processing of TYR in amelanotic melanoma cells by Western blotting after Endo H and PNGase F digestion, and following inhibition of proteasome activity. In contrast to the distribution and stability patterns of TYR in pigmented MNT-1 cells, the majority of TYR in amelanotic SK-MEL-28 melanoma cells was not correctly glycosylated, was trapped in the ER, and was quickly degraded by proteasomes. To clarify the stability and degradation of TYR in amelanotic melanoma cells, we also used metabolic labeling and immunoprecipitation. TYR had an extremely short half-life in amelanotic melanoma cells, and that stability could be markedly enhanced by treatment with proteasome inhibitors. The sum of these results suggests that the incorrect trafficking of TYR plays an important role in the disrupted pigmentation in amelanotic melanoma cells. In light of the role of TYRP1 in complexing with and stabilizing TYR in the ER (67), and of the fact that disruption of TYRP1 function results in abnormal proteasomal degradation of wild-type TYR in OCA3 cells (27), the lack of TYRP1 expression in SK-MEL-28 cells may also be an important factor resulting in the hypopigmentation of those cells similar to what occurs in OCA3.

Melanosomes are lysosome-related organelles, but their exact biogenesis is still poorly defined (29, 73). For instance, melanosomes and lysosomes contain many of the same structural proteins (e.g., LAMP, acidic hydrolases, v-ATPase, proton transport pumps (47, 74, 75)), and both are affected in several genetic disorders, such as the Chediak-Higashi and Hermansky-Pudlak syndromes (76, 77). The catalytic domains of TYR and other enzymes involved in melanogenesis are located within the lumen of the melanosomes, and it follows that their activity is likely to be dependent upon the intramelanosomal environment, including the pH (19). However, there is some controversy regarding the optimal pH for TYR activity. Melanosomes, like other lysosomal organelles, can be quite acidic, and it has been assumed that this low melanosomal pH facilitates melanosomal biogenesis (78). It has been suggested that human TYR activity is activated at acidic pH and that the enzyme is inactive at neutral pH (46). However, several independent groups have maintained that mammalian TYR has an optimal enzymatic activity that is near neutral pH and that its activity is gradually lost with decreasing pH (79–81). However, the pH at which melanin is produced and the pH at which the vesicular trafficking system works may function quite independently.

Baf and CCM, which at low concentrations specifically inhibit vATPases (51), have frequently been used to neutralize acidic compartments within cells. Mon, a proton ionophore, exchanges H+ for Na+ and has also been commonly used to neutralize acidic compartments. To clarify the effects of neutralizing the pH of acidic organelles on TYR processing and trafficking, we used immunofluorescence staining and Western blotting to examine the processing of TYR in amelanotic melanoma cells. TYR levels in amelanotic melanoma cells were dramatically increased following treatment with any of those protonophore or proton pump inhibitors. Surprisingly, the retention of TYR in the ER in amelanotic melanoma cells could be corrected by those agents (i.e., by increasing intracellular pH), which resulted in the enhanced transport of TYR from the ER to the Golgi. Thus, the abnormal acidification of intracellular organelles also plays an important role in the pathogenesis of hypopigmentation in amelanotic melanoma cells.

Although TYR was dramatically redistributed to stage II melanosomes in SK-MEL-28 cells following the neutralization of intracellular pH, TYR levels in endosomes were reduced in the presence of proton pump inhibitors but were increased in the presence of the protonophore. This suggests that the processing and trafficking of TYR in amelanotic melanocytes is related to the dysfunction of vATPases. vATPase was recently identified as a melanosomal protein (66), and this current study shows that it may play an important role in regulating pigmentation. Transport of TYR from the ER and its subsequent processing depend on the neutralization of pH in the Golgi, although the translocation of TYR to endosomes then requires the activation of vATPase. Neutralization of the pH within early melanosomes results in the accumulation of TYR in those organelles. Therefore, the activity of vATPases within intracellular organelles plays an important role in the sorting and function of TYR and in modulating pigmentation; dysfunction of that pH regulatory system may be responsible for the depigmented phenotype and pathogenesis of amelanotic melanocytes.

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REFERENCES

1. Spritz, R. A. (2000) Pigment Cell Res. 13, 15–20
2. Kashimoto, T., Valencia, J. C., Costin, G. E., Toyofuku, K., Watabe, H., Yasumoto, K., Rouzaud, F., Vieira, W. D., and Hearing, V. J. (2003) Pigment Cell Res. 16, 237–244
3. Shen, B., Rosenberg, M. B., and Orelow, S. J. (2001) Traffic 2, 202–211
4. Raposo, G., and Marks, M. S. (2002) Traffic 3, 237–248
5. Chapman, P. B. (1996) Immunol. Hum. Melanoma 12, 195–200
6. Rosenberg, S. A., and White, D. E. (1996) J. Immunother. 19, 81–84
7. Orelow, S. J., Silvers, W. K., Zhou, B. K., and Mintz, B. (1998) Cancer Res. 58, 1521–1523
8. Mendiratta, S. K., Thai, G., Eslahi, N. K., Thull, N. M., Matar, M., Bronte, V., and Pericel, F. (2001) Cancer Res. 61, 859–863
9. Takeuchi, H., Kuo, C., Morton, D. L., Wang, H.-J., and Hoon, D. S. B. (2003) Cancer Res. 63, 441–448
10. Tachibana, M. (2000) Pigment Cell Res. 13, 230–240
11. Busca, R., and Ballotti, R. (2000) Pigment Cell Res. 13, 60–69
12. Halaban, R., Bohm, M., Dotto, P., Moellmann, G., Cheng, E., and Zhang, Y. (1996) J. Invest. Dermatol. 106, 1366–1372
13. Yavuzer, U., Keenan, E., Lawings, P., Vachetzen, J., Currie, G., and Goding, C. R. (1995) Oncogene 10, 123–134
14. Du, J., Miller, A. J., Widjaja, W. H., Horstmann, M. A., Ramaswamy, S., and Fisher, D. E. (2003) Am. J. Path. 163, 333–343
15. Halaban, R., Cheng, E., Svedine, S., Aron, R., and Hebert, D. N. (2001) J. Biol. Chem. 276, 11933–11938
16. Izumi, K., Higashino, G. E., Pennella, R., Everett, M. A., and Fuller, B. B. (1993) J. Invest. Dermatol. 100, 806–811
17. Fuller, B. B., Inman, D. S., and Lunsford, J. B. (1988) J. Cell. Physiol. 134, 149–154
18. Burchill, S. A., Bennett, D. C., Holmes, A., and Thody, A. J. (1991) Pathobiology 59, 335–339
19. Ancans, J., Tobin, D. J., Hoogduijn, M. J., Smit, N. P., Wakamatsu, K., and Thody, A. J. (2001) Exp. Cell Res. 268, 26–35
20. Müller, G., Ruppert, S., Schmid, E., and Schütz, G. (1988) EMBO J. 7, 2723–2730
21. Kwon, B. S., Haq, A. K., Pomerantz, S. H., and Halaban, R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7473–7477
22. Ujvari, A., Aron, R., Eisenhaure, T., Cheng, E., Parag, H., Smicun, Y., Halaban, R., and Hebert, D. N. (2001) J. Biol. Chem. 276, 5924–5931
23. Körner, A., and Pawelek, J. M. (1982) Science 217, 1163–1165
24. Hearing, V. J. (1987) Methods Enzymol. 142, 154–165
25. Tripodi, R. K., Hearing, V. J., Uraha, K., Arceo, P., and Spritz, R. A. (1992) J. Biol. Chem. 267, 23707–23712
26. Negru, G., Dwek, R. A., and Petrescu, S. M. (2000) J. Biol. Chem. 275, 32200–32207
27. Toyofuku, K., Wada, I., Valencia, J. C., Kashimoto, T., Ferrans, V. J., and Hearing, V. J. (2001) FASEB J. 15, 2149–2161
28. Iozumi, K., Hoganson, G. E., Pennella, R., Everett, M. A., and Fuller, B. B. (1988) J. Biol. Chem. 263, 2193–2198
29. Ujvari, A., Aron, R., Eisenhaure, T., Cheng, E., Parag, H., Smicun, Y., Halaban, R., and Hebert, D. N. (2001) J. Biol. Chem. 276, 32200–32207
