Abnormal Acidification of Melanoma Cells Induces Tyrosinase Retention in the Early Secretory Pathway*

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Tyrosinase (monophenol monooxygenase, EC 1.14.18.1) is a copper-binding enzyme that catalyzes the oxidation of monohydric and dihydric phenols (catechols) to their corresponding quinones, the rate-limiting reaction in melanin synthesis (1, 2). Peptides derived from tyrosinase are frequently presented on melanoma cells by major histocompatibility molecules (3, 4). The development of immunotherapies for patients with melanoma is based in part on employing cytotoxic T-cell recognizing peptides as the immunogen (5). The production of antigenic peptides involves an accumulation of tyrosinase in the endoplasmic reticulum (ER) as a monophenol monooxygenase (EC 1.14.18.1) is a copper-binding enzyme that catalyzes the oxidation of monohydric and dihydric phenols (catechols) to their corresponding quinones, the rate-limiting reaction in melanin synthesis (1, 2). Peptides derived from tyrosinase are frequently presented on melanoma cells by major histocompatibility molecules (3, 4). The development of immunotherapies for patients with melanoma is based in part on employing cytotoxic T-cell recognizing peptides as the immunogen (5). The production of these peptides is contingent on the presence of tyrosinase and its proteolytic degradation products. However, tyrosinase peptide presentation is an aberrant phenotype of melanoma cells, because tyrosinase in normal melanocytes is a stable enzyme that is localized to the melanosomes, the site of melanin synthesis.

The production of antigenic peptides involves an accumulation of tyrosinase in the endoplasmic reticulum (ER) as a 70-kDa high mannose glycoform and its subsequent routing to the cytoplasm for degradation by the proteasome (6, 7). Failure of tyrosinase in these melanoma cells to be processed in the medial Golgi as indicated by endoglycosidase H (Endo H) digestion and confocal microscopy (6) is reminiscent of albinism and is a feature of tyrosinasePositive melanoma tumors may indicate the onset of enhanced glycolysis and extracellular acidification, conditions known to favor metastatic spread and resistance to weak base chemotherapy drugs.

In tyrosinase-positive amelanotic melanoma cells, inactive tyrosinase accumulates in the endoplasmic reticulum. Based on studies described here, we propose that aberrant vacuolar proton ATPase (V-ATPase)-mediated proton transport in melanoma cells disrupts tyrosinase trafficking through the secretory pathway. Amelanotic but not melanotic melanoma cells or normal melanocytes display elevated proton export as observed by the acidification of the extracellular medium and their ability to maintain neutral intracellular pH. Tyrosinase activity and transit through the Golgi were restored by either maintaining the melanoma cells in alkaline medium (pH 7.4–7.7) or by restricting glucose uptake. The translocation of tyrosinase out of the endoplasmic reticulum and the induction of cell pigmentation in the presence of the ionophore monensin or the specific V-ATPase inhibitors concanamycin A and bafloymycin A1 supported a role for V-ATPases in this process. Because it was previously shown that V-ATPase activity is increased in solid tumors in response to an acidified environment, the appearance of hypopigmented cells in tyrosinasePositive melanoma tumors may indicate the onset of enhanced glycolysis and extracellular acidification, conditions known to favor metastatic spread and resistance to weak base chemotherapy drugs.

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nucleus (22–24). Increased V-ATPase activity has been implicated in the acidification of endosomes, trans-Golgi network, and lysosomes in solid tumors to accommodate the acidic environment (25–27). Substances that cause alkalization of vesicular compartments such as the Na+/K+ and proton ionophore monensin or the V-ATPase-specific inhibitors concanamycin A or bafilomycin A1 also induce the release of chemotherapeutic drugs and enhance their accumulation in the nucleus (28). Because tyrosinase activity can be suppressed by acidified conditions (2, 29), we explored the possibility that increased proton pump activity also affects tyrosinase activity and processing in melanoma cells. We show here that tyrosinase trafficking and activity in amelanotic melanoma cells were restored after alkaline treatment or inhibition of V-ATPase activity. The data support the hypothesis that protonation, possibly in the ER-Golgi interface, disrupts tyrosinase maturation in melanoma cells resulting in the amelanotic phenotype, tyrosinase degradation, and antigen production.

MATERIALS AND METHODS

Cell Culture—Normal human melanocytes were cultured from newborn foreskin fibroblasts in F-10 medium supplemented with glutamine (2 mM), penicillin-streptomycin (100 units/ml), and 7% fetal bovine serum (all from Invitrogen) termed basal medium, which was further enriched with several ingredients required for normal melanocyte proliferation. These ingredients included 85 nM 12-O-tetradecanoylphorbol-13-ace-tate, 0.1 mM isobutylmethylxanthine, 2.5 mM cholera toxin, 1 μM NaN3, and 0.1 mM Nα,N′,O′-dibutyryladenosine-3′-AMP (β,γcAMP) (TICOY, Sigma) (30).

Human metastatic amelanotic melanoma cells (YUGEN5, 501 mel, YUSITI, and YUSAC2) (30), were maintained in the Ham’s F-10 basal medium. The melanotic Hei178 cells were grown in the Ham’s F-10 basal medium supplemented with growth factors (2 ng/ml fibroblast growth factor 2, 10 ng/ml endothelin 1, 10 ng/ml hepatocyte growth factor plus 0.2 ng/ml heparin) and used during the second passage in culture. The melanotic MNT1 melanoma cells (31) (obtained from Dr. M. S. Marks, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA) were grown in Dulbecco’s modified Eagle’s medium plus 20% serum and 10% AIM-V medium (Invitrogen) as described previously (32). When needed, the extracellular pH (pHe) of the medium was monitored daily and adjusted to pH 7.7 with 1 N NaOH. Cells were incubated in the basal medium, unmodified OptiMEM (Invitrogen) plus 2% fetal bovine serum, Dulbecco’s modified Eagle’s glucose-free medium, or RPMI 1640 medium selectively amine (Invitrogen) reconstituted with sodium pyruvate, tyrosine, and glucose or galactose as indicated. In some experiments, the medium was supplemented with 50 μM freshly prepared DOPA (1 mM stock solution in PBS, Sigma), monensin (10 μM stock solution in Me2SO, Sigma), Aβ or bafilomycin A1 (20 μM stock solution dissolved in Me2SO, both from Calbiochem), pepstatin (10 mg/ml stock solution), or leupeptin (20 mg/ml stock solution, Sigma) using Me2SO as a control when needed.

Western blot Analysis, Precipitation, and Antibodies—CHAPS lysis buffer (2% CHAPS in 50 mM HEPES and 200 mM NaCl, pH 7.5) containing protease inhibitors (Complete Protease Inhibitor mixture, Roche Molecular Biochemicals) was used to lyse cells and wash bead-bound precipitated material as described previously (6). Western blot analyses were performed on whole cell lysates (40 μg of protein/lane as measured by the Bio-Rad protein assay reagent, Bio-Rad), anti-tyrosinase immunoprecipitated proteins (C-19 goat, Santa Cruz Biotechnology, Santa Cruz, CA), or affinity-purified glycoproteins using wheat germ agglutinin (WGA) bound to beads (leitin from Triticum vulgaris) following standard procedures or the manufacturer’s instructions (Sigma). Endo H (Roche Molecular Biochemicals) digestion of precipitated proteins was performed as described previously (6, 11). Tyrosinase was detected with mouse mAb Ts31 (33), and protein loading in each lane was assessed by staining the gels with Coomassie Brilliant Blue after transfer of the proteins to membranes and by immunoblotting with anti-tyrosinase antibodies (anti-T311, 33).

Tyrosinase Activity—Tyrosinase assays were performed with [l-3,5,3H]tyrosine (PerkinElmer Life Sciences) as a substrate (34–36). Reaction mixtures (200 μl of final volume) containing 150 μg of cell extract protein prepared in 2% CHAPS buffer, 50 μM l-tyrosine, 1 μCi/assay l-3,5,3H]tyrosine, and 50 μM l-DOPA were incubated for 60 min at 37°C. Reactions were stopped with 200-μl solution of 10% activated charcoal in 0.1 M citric acid (w/v), the charcoal slurries passed through Dowex columns (350 μl), and radioactivity of the eluate in scintillation fluid was measured with a scintillation counter. One unit of tyrosinase was defined as the amount of enzyme that catalyzed the oxidation of 1 mmol tyrosine in 1 min. All reactions were performed in triplicates or quadruplicates, and the standard errors were in the range of 15% total counts.

Intracellular pH (pHi) Measurements—Normal and malignant melanocytes were grown on 18-mm glass coverslips precoated with a 1:1 mixture of collagen I and fibronectin-like RGD fragments at a final concentration of 50 μg/ml for 48–72 h. Cells were then incubated with the pH-sensitive dye BCECF AM at a final concentration of 5 μM for 4 min at pH 7.3 at room temperature and 5% CO2, as described previously (37, 38). Following a medium change to pH 7.3 or 7.0, the cells were maintained for an additional 20 min at 37°C, 5% CO2 to allow complete hydrolysis of the dye ester. The plates were then mounted on the microscope stage, and pHi was monitored at 37°C under humidified air containing 5% CO2. Cellular pHi values were calculated based on data from whole excitation spectra (37, 38). All measurements were done in triplicates.

Metabolic Labeling—Pulse-chase experiments were performed as described previously (8). Cells were pulse-labeled for 15 min with [35S]Met/Cys (0.7 μCi/ml, EasyTag, PerkinElmer Life Sciences) in methionine/cysteine-free RPMI 1640 medium (Invitrogen) and either collected immediately or after chase incubation with non-radioactive medium (Ham’s F-10 medium) for the indicated period of time. Experiments were performed in medium supplemented with tyrosine as indicated and then subjected to immunoprecipitation with rabbit anti-tyrosinase antibodies. Following extensive washing with radioimmune precipitation buffer, half of the precipitated products were digested with Endo H overnight. Eluted proteins were fractionated in SDS-PAGE, and dried gels were analyzed by autoradiography. Densities of radiolabeled tyrosinase bands on the x-ray films were determined using a Molecular Dynamics PhosphorImager.

Immunofluorescence Microscopy—Melanoma cells were grown on chamber slides in unmodified Ham’s F-10 medium at pH 7.4. Treated and untreated cells were washed with PBS, fixed in 4% formaldehyde/PBS, and permeabilized with 0.1% Triton X-100/PBS. The permeabilized and fixed cells were then incubated with antibodies against tyrosinase (C-19 goat), ERGIC-53 (mAb, a gift from Dr. H.-P. Hauri, Geneva, Switzerland; a marker of ER–ER Golgi intermediate compartment), COPI (rabbit polyclonal anti-β-COPI antibodies, a gift from Dr. G. Warren, Department of Cell Biology, Yale University, New Haven, CT; a marker for ER-ERGIC-Golgi compartments), or calnexin (mAb, StressGen Biotechnologies Corporation, Victoria, British Columbia, Canada; a marker for quality control of newly synthesized glycoproteins). Cells were incubated with fluorescein anti-goat conjugates (Santa Cruz Biotechnology) or rhodamine anti-mouse or anti-rabbit conjugates (Molecular Probes, Eugene, OR). All dilutions were in 0.1% bovine serum albumin/PBS. Indirect immunofluorescence visualization was performed with an inverted Bio-Rad MRC-600 laser confocal microscope. Images were processed with Bio-Rad confocal assistant software.

RESULTS

Stabilization of Tyrosinase by Raising Extracellular pH—Under normal conditions, the medium from amelanotic melanoma cells becomes rapidly acidified within a day of medium change (pH 7.0–7.2), whereas that of normal melanocytes or the pigmented metastatic melanoma Hei178 remains basic (pH 7.4–7.5). Therefore, we evaluated pigmentation and tyrosinase processing in YUGEN5 melanoma cells subcultured in Ham’s F-10 medium, adjusted daily to pH 7.7, and compared it to cells that were continuously grown in unmodified Ham’s F-10 medium. Alkalization of the pHe had a marked effect as the cells became pigmented during 2 weeks of culture (Fig. 1A). An analysis of steady-state tyrosinase levels demonstrated an increase in the abundance of tyrosinase protein (Fig. 1B, lane 1 with 2, arrow) (6).

The identification of the higher molecular weight tyrosinase glycoform as a protein that had been modified by Golgi enzymes was verified by Endo H digestion. Endo H cleaves N-
linked oligosaccharides between the two N-acetylglucosamine residues in the core region of the oligosaccharide chain of high mannose but not complex carbohydrates. Because the addition of complex sugars occurs in the medial Golgi, the limited substrate specificity of this enzyme provides a useful tool for monitoring the subcellular location of glycoproteins. Endo H treatment had only a slight effect on the electrophoretic mobility of mature tyrosinase (Fig. 1B, lanes 4 and 6, solid arrow) indicating that the majority of the seven tyrosinase N-linked glycans had been modified with complex sugars in the Golgi (39). In contrast, the faster migrating immature glycoform (Fig. 1B, bands marked with empty arrow) was digested to its 58-kDa polypeptide indicative of complete sensitivity of all of seven glycans in tyrosinase, a characteristic of ER or cis-Golgi residency (Fig. 1B, lanes 4 and 6, band marked DG). Species of low molecular mass of ∼58 kDa (Fig. 1B, lane 2 marked with spearhead) also accumulated after incubation in alkaline pH. This protein band represents the non-glycosylated tyrosinase as shown by its inability to bind WGA (Fig. 1B, lanes 5) as reported previously (6, 11).

Further evidence that alkaline pH promoted tyrosinase maturation and activation was obtained by analyzing the effect of DOPA on this process. We have previously shown that the addition of the cofactor DOPA to the growth medium in the presence of catalytic amounts of the substrate tyrosine promoted tyrosinase activation and maturation in melanoma cells within hours (11). Because DOPA activation of tyrosinase is dependent on pH (2, 29), we tested whether low pH could suppress the DOPA effect. Indeed maintaining the cells at low pH hampered the DOPA/tyrosine-induced maturation (Fig. 1C, compare lanes 1–3 with 4–7), suggesting that long term growth in acidified pH suppresses DOPA activation, tyrosinase maturation, and pigmentation.

Tyrosinase Processing Is Induced by Glucose Restriction in Melanoma Cells—Because increased glucose consumption can increase acidity as a result of the accumulation of lactic acid in the cell environment (17), we tested whether eliminating glucose in the medium could affect pigmentation, tyrosinase activity, and maturation. Toward this end, melanoma cells (YUGEN8 and YUSIT1) were grown for 3 days in glucose-free medium with 1 mM pyruvate supplemented with 25 mM glucose (+) or 2 mM galactose (−). A, melanoma cell pellets (YUGEN8 and YUSIT1) after 3 days of incubation in the experimental medium, B, time course analysis of tyrosinase in response to glucose deprivation. YUGEN8 melanoma cells were grown in galactose-supplemented Dulbecco’s modified Eagle’s glucose-free medium for increasing periods of time. Cell extracts were subjected to Western blot analysis first with anti-tyrosinase T311 mAb (TYR) and then with anti-actin antibodies (actin). C, DOPA-stimulated tyrosinase activity in cell extracts of YUGEN8 melanoma cells grown in the presence or absence of glucose as shown in A. D, maturation of tyrosinase in glucose-free medium supplemented with different concentrations of tyrosine. Western blots with T311 anti-tyrosinase mAb of whole cell lysates derived from YUGEN8, YUSIT1, and 501 mel melanoma cells grown for 3 days in glucose-free or glucose-supplemented medium with 10 or 100 μM tyrosine.
Tyrosinase Misfolding by Defective Acidification

FIG. 3. Amelanotic melanoma cells maintain high pH values after acidification. The pH of normal human melanocytes (■), amelanotic YUGEN8 (●) and 501 mel (○) melanoma cells, and melanotic Heik178 (■) and MNT1 (○) melanoma cells was measured after exposure to pH 7.0. Measurements were done in growth medium at pH 7.3, 10 min before pH changes (~10 data point) and up to 60 min after a shift to pH 7.0 (acidification). Reduction in pH during initial recording before time 0 is sometimes seen when the lost CO2 during transfer to the microscope was not completely reassimilated. A shift to pH 7.3 did not cause a change in intracellular pH in any of the cell lines. At least five consecutive measurements were taken on each of three fields. Data are means of triplicate measurements in a representative experiment of three experiments, and error bars indicate the means ± S.D. Dashed and solid lines indicate amelanotic and melanotic cell lines, respectively.

consumption (Fig. 2) could be modulated by the concentrations of tyrosine in the medium. As the glucose-free medium used above contained a high tyrosine concentration (400 μM), we incubated three melanoma cell lines, YUGEN8, 501 mel, and YUSIT1, in RPMI 1640 select™-amine medium supplemented with glucose or galactose in the presence of low (10 μM) or high (100 μM) tyrosine for 3 days. Tyrosinase was processed to the 80-kDa form in cells grown in glucose-free medium at both low and high tyrosine concentrations in melanoma cells exhibiting relatively modest levels of tyrosinase (Fig. 2D, YUGEN8, compare lane 1 with 2 and lane 3 with 4, solid and empty arrows). Melanoma cells with lower levels of tyrosinase displayed different thresholds of activation. The 501 mel cells required at least 100 μM tyrosine for stabilization (Fig. 2D, compare lane 6 with 8), whereas the enzyme remained as the ER 70-kDa glycoform in YUSIT1 melanoma cells even at 100 μM tyrosine (Fig. 2D, lanes 9–12, empty arrow). Therefore, in agreement with previous observations (11), the effectiveness of glucose restriction and alkaline extracellular pH was dependent on the concentration of tyrosine in the medium and the levels of endogenous tyrosinase, suggesting that the maturation process was dependent on tyrosinase activity.

Amelanotic Melanoma Cells Maintain Higher Intracellular pH Values—The enhanced extracellular acidification observed for amelanotic melanoma cells indicated an increased proton pump activity at their plasma membrane. In addition, the surface proton pumps, Na+/H+ antiporters and Cl−/HCO3− exchangers are known to be activated in tumor cells to maintain pH and protect the cells from the acidic extracellular environment (reviewed in Ref. 28). Therefore, we assessed the pHi in response to external acidification in normal melanocytes and compared it with melanotic and amelanotic melanoma cells.

A shift to extracellular pH 7.0 induced a dramatic drop in intracellular pH in normal melanocytes and pigmented melanoma cells (Heik178 and MNT1). In contrast, the pHi of the amelanotic melanoma cells (YUGEN8 and 501 mel) remained relatively alkaline and was persistently 0.4–0.5 pH units above that of cells that retained their pigmented phenotype in culture (normal and malignant melanocytes), indicating high compensating proton pump activity in amelanotic melanoma cells (Fig. 3). Changes in the activity of the proton pumps in melanoma cells in response to growth in low extracellular pH for several days were recently reported (16).

Inhibition of Proton Pump Activity Promotes Tyrosinase Maturation—Activation of proton pumps in cultured amelanotic melanoma cells can be inferred from the highly acidified conditioned medium and their ability to compensate their pHi when exposed to pH 7.0. V-ATPases have been implicated in neutralizing cytosolic pH by pumping protons away from the cytoplasm to the outside milieu as well as into acidic organelles such as the Golgi, endosomes, and lysosomes (25, 40, 41). Therefore, we tested the effect of the Na+/K+ and proton ionophore monensin known to reversibly raise the pH of endocytic vesicles (22, 42) and the high affinity V-ATPase inhibitors concanamycin A and bafilomycin A1 (43) on tyrosinase maturation and activity. Four different strains of amelanotic melanoma cells became highly pigmented within 2 h of incubation with each of these compounds in the presence of 100 μM tyrosine without any manipulation of the extracellular pH (data not shown). In agreement with published observations (44, 45), the three agents also increased the level of pigmentation of the already highly melanized normal human melanocytes derived from Caucasian donors. The increase in pigmentation in all cell types was probably because of an increase in in situ tyrosinase activity in response to alkalization of vesicular compartments known to be acidified under normal conditions (46, 47).

In vitro tyrosinase activity of cell extracts from normal melanocytes and melanoma cells (YUGEN8 and 501 mel) increased 5- and 2-fold in a pH-dependent manner between pH 6.6 and 8.0, respectively, suggesting that mature and immature forms of tyrosinase are activated at basic pH.

Analyses of steady-state tyrosinase levels showed that in normal human melanocytes, the mature 80-kDa enzyme, the predominant glycoform, was not affected by monensin, concanamycin A, or bafilomycin A1 (Fig. 4A, compare lane 1 with 2–4). In contrast, the treatment of melanoma cells with nanomolar concentrations of the two V-ATPase inhibitors induced the conversion of the 70-kDa glycoform to the mature 80-kDa glycoform (Fig. 4A, lanes 7, 8, 11, 12, 15, 16, 19, and 20). Although high concentrations of tyrosine were not required to elicit maturation (Fig. 4A, lanes 17–20, B, as indicated, and C and D), higher levels of tyrosine enhanced the effect of the V-ATPase inhibitors on tyrosinase (Fig. 4B). Dose response analysis showed that concanamycin A and bafilomycin A1 were optimally effective at 20 and 50 nM, respectively. However, 5 nM of each inhibitor was sufficient to induce tyrosinase maturation (Fig. 4C). At optimal concentrations, high molecular weight forms of tyrosinase began to accumulate within 30 min of incubation with maximum effect reaching within 3 h (Fig. 4D).

The low concentration required to elicit an effect and the higher effectiveness of concanamycin A over bafilomycin A1 on tyrosinase maturation are indicative of specific inhibition of V-ATPase and in agreement with the relative potency of each compound toward V-ATPase inhibition (45).

Interestingly, despite a marked increase in pigmentation (data not shown), monensin did not affect the SDS-PAGE migration pattern and abundance of tyrosinase (Fig. 4A, lanes 6, 10, 14, and 18). Modification of tyrosinase in the Golgi in response to the V-ATPase inhibitors but not after monensin treatment was further confirmed by the accumulation of the 80-kDa mature tyrosinase (Fig. 5A, lanes 1–8, compare bands

2 R. Halaban, E. Cheng, and D. N. Hebert, unpublished results that are in agreement with the findings published in Ref. 44.
Inhibitors of V-ATPase enhance tyrosinase maturation. A, Western blot analyses for tyrosinase (TYR) normalized to actin using whole cell lysates derived from normal melanocytes (NM) and melanoma cells (YUGEN8, 501 mel, and YUSIT1) or WGA-bound glycoproteins from YUSAC2 melanoma cells. The various cell types were incubated for 4 h before harvest in medium supplemented with the diluent 1 µM Me2SO (DMSO), 10 µM monensin (Mon), concanamycin A (CCM), or bafilomycin A1 (Baf) at 100 nM each. Ham’s F-10 medium supplemented with low (10 µM, lanes 1–4) or high tyrosine (100 µM, lanes 5–20) was used. Solid and empty arrows indicate mature and immature unprocessed tyrosinase proteins, respectively. B, high concentration of tyrosine in the medium enhanced the concanamycin A-induced tyrosinase maturation in melanoma cells. YUGEN8 cells were harvested after 4-h incubation in Ham’s F-10 medium with low (10 µM) or high (100 µM) tyrosine in the absence and presence of concanamycin A (100 nM). C, dose response of YUGEN8 melanoma cells to CCM and Baf supplemented to Ham’s F-10 medium (10 µM tyrosine). Cells were harvested after incubation in the experimental medium for 4 h. D, kinetics of tyrosinase maturation in melanoma cells. YUGEN8 melanoma cells were incubated in unmodified Ham’s F-10 medium (10 µM tyrosine) supplemented with CCM (20 nM) or Baf (50 nM) for increasing duration.

FIG. 4.

To determine whether tyrosinase stabilization by alkalization or inhibition of V-ATPases could be attributed to the interference with lysosomal proteolysis, tyrosinase was monitored after inhibition of the lysosomal proteases with leupeptin and pepstatin. Treatment of melanoma cells with leupeptin and pepstatin caused only a slight increase in the levels of steady-state or newly synthesized tyrosinase with complex carbohydrates in YUGEN8 (Fig. 5A, lanes 9–12), but not in 501 mel cells (Fig. 5A, lanes 17–20). The results indicated that although tyrosinase in small amounts was able to reach a post-Golgi compartment in some melanoma cell strains, lysosomal protease inhibition could not account for the pigmentatin and enhanced maturation of tyrosinase observed after alkalization or V-ATPase inhibition.

Exit of Tyrosinase from the ER in Response to Intracellular Alkalization—Confocal immunofluorescence analyses indicated that tyrosinase remained in the ER in melanoma cells marked with empty and solid arrows, respectively. The simultaneous addition of monensin and concanamycin A prevented the concanamycin A-induced tyrosinase maturation (Fig. 5A, lanes 9–12), suggesting a block in the ER, ERGIC, or cis-Golgi, because monensin blocks trafficking in a pre-Golgi compartment without interfering with Golgi enzymes (48–50). Pulse-chase experiments confirmed that concanamycin A and bafilomycin A1 but not monensin enhanced the Golgi modification of newly synthesized tyrosinase in melanoma cells (Fig. 5B, YUGEN8, lanes 1–12, 17–20, bands marked with arrow).

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To determine whether tyrosinase maturation by alkalization or inhibition of V-ATPases could be attributed to the interference with lysosomal proteolysis, tyrosinase was monitored after inhibition of the lysosomal proteases with leupeptin and pepstatin. Treatment of melanoma cells with leupeptin and pepstatin caused only a slight increase in the levels of steady-state or newly synthesized tyrosinase with complex carbohydrates in YUGEN8 (Fig. 5A, lanes 9–12), but not in 501 mel cells (Fig. 5A, lanes 17–20). The results indicated that although tyrosinase in small amounts was able to reach a post-Golgi compartment in some melanoma cell strains, lysosomal protease inhibition could not account for the pigmentatin and enhanced maturation of tyrosinase observed after alkalization or V-ATPase inhibition.

FIG. 5. Golgi modification of tyrosinase in response to V-ATPase and protease inhibitors. A, steady-state tyrosinase as revealed by anti-tyrosinase Western blotting. Cells were treated with 1 µM Me2SO (DMSO), 20 µM monensin (Mon), 20 nM concanamycin A (CCM), 50 nM bafilomycin A1 (Baf), or 10 µg/ml leupeptin and 25 µg/ml pepstatin (Leu/Pep) for 3 h. Tyrosinase was precipitated from melanoma cell extracts (YUGEN8 and 501 mel) with WGA-bound beads. Endo H-digested (lanes 1–8) or non-digested (lanes 9–12) proteins were subjected to SDS-PAGE and Western blotting. Alternatively, immunoprecipitated tyrosinase (C-19 antibodies) was subjected to treatment with or without Endo H (lanes 13–20). Solid and empty arrows indicate mature and immature tyrosinase, respectively. Band marked is the deglycosylated (DG) tyrosinase polypeptide. Note that the x-ray film representing lanes 17–20 was overexposed to rule out the presence of any minor bands. B, autoradiogram of metabolically radiolabeled tyrosinase immunoprecipitated from YUGEN8 melanoma cells incubated in Ham’s F-10 medium containing 100 µM tyrosine with inhibitors noted above. Cells were metabolically labeled with [35S]Met/Cys for 15 min and harvested immediately (0 h) or after a 3-h chase in non-radioactive medium (3 h). The indicated agents were present during the 2-h starvation in Cys/Met-free medium.

FIG. 6. Tyrosinase is not present in the ERGIC or cis-Golgi in melanoma cells. Immunofluorescence confocal microscopy images are shown with immunostaining of tyrosinase (green) and ERGIC-53 or COPI (red) in normal human melanocytes (NM) and melanoma cells (YUGEN8). The panels on the right display merged images. Notice that tyrosinase is spread out in regions that do not include the ERGIC or cis-Golgi in melanoma cells. The non-overlapping red punctated vesicles are particularly obvious above the nucleus of YUGEN8 melanoma cells stained with either ERGIC-53 or COPI (MERGE).
under steady-state conditions (Fig. 6). Antibodies to ERGIC-53 and COPI stained normal melanocytes and melanoma cells in a characteristic perinuclear crescent shape pattern representing the Golgi as well as in punctate structures peripheral to the ER region corresponding to the ERGIC and the ER. In normal melanocytes, only partial overlap was seen between tyrosinase and ERGIC-53 or COPI (Fig. 6, NM). In contrast, in the melanoma cells, tyrosinase colocalized with ERGIC-53 and COPI in the ER region but not in the ERGIC or Golgi regions as shown by the red rhodamine vesicles containing ERGIC-53 or COPI that did not merge with green fluorescein isothiocyanate-tyrosinase (Fig. 6, YUGENs). Therefore, if tyrosinase travels beyond the ER in untreated melanoma cells, its presence there must be short-lived, as it cannot be detected under steady-state conditions by immunostaining.

Simultaneous immunostaining with tyrosinase and the ER marker calnexin showed that tyrosinase exited from the ER in response to concanamycin A and monensin (Fig. 7). Within 1-h treatment with concanamycin A, tyrosinase appeared in structures that did not coincide with the ER (Fig. 7, CCD, 1 h). After 2-h treatment, tyrosinase localized in tubules extending from perinuclear compartments across the cell (Fig. 7, CCD, 2 h). Tyrosinase was also distributed outside the ER and did not colocalize with calnexin after a 2-h incubation with monensin (Fig. 7, Mon). The confocal images confirmed the steady-state immunoblotting results demonstrating exit of tyrosinase from the ER in response to imposed alkalization. Although the nature of the tyrosinase-positive post-ER structures after monensin and concanamycin A treatment has yet to be determined, it is possible that the enzyme was exported to distant sites by two different pathways, one that involved the Golgi medial-processing compartment and the other that did not.

**Fig. 7. Tyrosinase export from the ER is induced by V-ATPase inhibition.** Forced pH changes across vacuolar compartment-induced export of tyrosinase to distal sites. Immunofluorescence confocal microscopy was performed on YUGENs melanoma cells grown in Ham’s F-10 medium after treatment with 1 μM Me2SO (DMSO) for 2 h, 10 μM monensin (Mon) for 2 h, or 20 nM concanamycin A (CCM) for 1 or 2 h as indicated. The left green panels represent tyrosinase (TYR) detected with anti-tyrosinase antibodies (C-19), the red middle panels show the localization of the ER resident protein calnexin (CNX), and the right panels display merged images in yellow (Merge).

**DISCUSSION**

We provided evidence that abnormal acidification of the extracellular milieu is the probable reason for the decline in tyrosinase catalytic activity in tyrosinase-positive amelanotic melanoma cells. In these cells, tyrosinase catalytic activity was restored by alkalization in a manner dependent on the presence of extracellular tyrosine. Alkalization was accomplished by adjusting the pH of the extracellular medium, by glucose deprivation, or by using agents that inhibit vacuolar proton ATPases or dissipate pH gradients across membranes. Therefore, the inactivation of tyrosinase is likely to be the consequence of increased proton pump activity in the malignant cells compared with normal melanocytes. This change was also reflected by the appearance of higher pH under steady-state conditions in melanoma cells. The higher proton pump activity is probably localized at both the cell membrane and within the endomembranes. Whereas Na+/H+ antiporters and Cl/ /HCO3- exchangers are known to be activated in tumor cells in order to maintain pH, V-ATPases are the major proton pumps of vesicular compartments (reviewed in Ref. 24). V-ATPases play a principle role in generating and maintaining the acidic environment in the lumen of intracellular organelles such as the Golgi, endosomes, and lysosomes (reviewed in Refs. 51–54).

Although the Golgi contains active V-ATPases, the ER does not (47, 55, 56). Therefore, tyrosinase may encounter an increased activity of this proton pump in a compartment anterograde to the ER in melanoma cells.

The ionophore monensin and two V-ATPase inhibitors allowed the release of tyrosinase from the ER and induced pigmentation, yet only the V-ATPase inhibitors promoted tyrosinase acquisition of complex sugars. Monensin and the V-ATPase inhibitors affect luminal processes at the ER-Golgi boundary via different mechanisms. Monensin blocks ER trafficking of glycoproteins such as IgG and transferrin in a pre-Golgi compartment without interfering with Golgi enzymes (48–50). On the other hand, bafilomycin A1 inhibits retrograde transport of proteins such as ERGIC-53 from the pre-Golgi-compartment back to the ER but not the anterograde transport of proteins from the ER to the Golgi (55). Because both agents cause alkalization of subcellular organelles, tyrosinase exit from the ER and pigmentation was probably enhanced because of activation of enzymatic activity by the increased pH. *In vitro* tyrosinase activity employing cell extracts from normal and malignant melanocytes expressing mature and immature forms, respectively, showed a pH-dependent activity with tyrosinase being 2–5-fold more active at pH 8.0 compared with pH 6.3 (data not shown). Therefore, like in vitro, the *in vivo* activity did not require modification to complex carbohydrates.

This conclusion is consistent with published values of pH within the secretory pathway and their changes in response to V-ATPase inhibition (46, 48). The ER and the Golgi maintain a pH of 7.2 ± 0.2 and 6.4 ± 0.3, respectively, and bafilomycin A1 induced alkalization of the various regions of the Golgi complex but did not affect the pH of the ER (57). Therefore, the aberrant accumulation of tyrosinase in the ER of melanoma cells raises the possibility that the acidified ER-Golgi boundary of melanoma cells is hostile to tyrosinase maturation. Even small changes in luminal pH can cause a significant change in protein processing and activation as shown for the processing of adrenocorticotropic hormone from its pro-opiomelanocortin precursor (58).

Quality control processes that monitor the fidelity of the maturation process appear to be in place throughout the secretory pathway (59). Some misfolded or partially assembled proteins that have escaped the ER can still be subjected to quality control in the early secretory system, because they can be
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retrieved from post-ER compartments back to the ER through COPI vesicles (60–62). In these cases, the inhibition of the COPI retrieval system induced the accumulation of the respective protein in post-ER compartments.

In light of these observations, the accumulation of tyrosinase in the ER of melanoma cells might also be the result of the quality control system in the ERGIC-cis-Golgi. Tyrosinase under steady-state conditions colocalized with the ER marker calnexin and with the ER portion of ERGIC-53 and COPI, suggesting a rapid retrograde transport to the ER if it reached a post-ER-pre-Golgi compartment. The observations that melanin, even in the presence of concanamycin A, elicited tyrosinase activation, ER exit, transport to distant sites, and pigmentation in the absence of Golgi processing, suggesting that melanin acts by dissipating a pH gradient upstream of the concanamycin A-affect ed site such as the ERGIC. These results also demonstrate that basic pH is sufficient to activate tyrosinase, which is in agreement with the pH-dependent in vitro tyrosinase activity, and that the addition of complex oligosaccharides is dispensable for tyrosinase activity.

The concept of abnormal acidification of intracellular organelles including the melanosomes as the cause for an amelanotic phenotype is supported also by genetic evidence. Oculocutaneous albinism 2 is an inherited condition in which individuals suffer loss-of-function mutation in the P-protein (77). The P-protein is an integral membrane protein (68) that is required for the proper functioning of the melanosomal maturation machinery. Individuals suffering from oculocutaneous albinism 2 have a defect in the melanosomal maturation pathway, which is in agreement with the pH-dependent tyrosinase activity, because the pH-dependent tyrosinase activity is a consequence of these metabolic pathways.

In light of these observations, the accumulation of tyrosinase in melanoma cells is unaffected (77).

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