THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 17, Issue of April 26, pp. 14821–14828, 2002
Printed in U.S.A.
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

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

Received for publication, December 3, 2001, and in revised form, January 10, 2002
Published, JBC Papers in Press, January 25, 2002, DOI 10.1074/jbc.M111497200

Ruth Halaban‡§, Robin S. Patton¶, Elaine Cheng‡, Sherri Svedine¶, E. Sergio Trombetta‖, Miriam L. Wahl, Stephen Ariyan‡‡, and Daniel N. Hebert¶§§**

*From the Departments of ‡Dermatology, ‖Cell Biology, and ‡‡Plastic Surgery, Yale University School of Medicine, New Haven, Connecticut 06520, the ¶Department of Biochemistry and Molecular Biology, Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003, and the **Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107*

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 bafilomycin 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 tyrosinase-positive melanoma tumors may indicate the onset of enhanced glycolysis and extracellular acidification, conditions known to favor metastatic spread and resistance to weak base chemotherapeutic drugs.

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 tyrosinase 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)[1] 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 albino mutant forms of tyrosinase that contain loss-of-function mutations and are retained in the ER (8–10). The observations that incubating melanoma cells with the cofactor DOPA or high concentrations of the substrate tyrosine enhanced the exit of tyrosinase from the ER, its carbohydrate modification in the Golgi, and transport to the melanosomes and melanin production suggested that tyrosinase inactivation is associated with aberrant misfolding and ER retention (11). However, the cause of inactivation of non-mutated tyrosinase in melanoma cells has not yet been elucidated.

We reasoned that inactivation of tyrosinase is probably linked to tumor-induced metabolic changes. A common phenotype shared by melanoma cells is acidification of the extracellular milieu and poor response to chemotherapy (12–15). Melanoma cells adapted to grow under hypoxic conditions acidify their immediate extracellular environment because of high rates of glucose uptake, increased glycolysis, and the accumulation of lactic acid (12, 16–18), a process known as the Warburg effect (19, 20). In fact, increased glucose uptake is currently the basis for melanoma tumor staging by PET (^{18}F-fluorodeoxyglucose positron emission tomography) (21). These metabolic changes are likely to contribute to the drug-resistance phenotype, because the extent of multidrug resistance in advanced melanoma lesions does not correlate with the expression of P-glycoprotein, multidrug resistance-1, even after chemotherapeutic treatment (13–15).

An alternative mechanism shared by drug-resistant cells is sequestration of weak base chemotherapeutics in acidic organelles away from their sites of action in the cytosol and

* This work was supported by U. S. Public Health Service Grants AR39848 and CA44542 (to R. H.), AR41942 (to R. E. Tigelaar, Yale Skin Diseases Research Center), and CA79864 (to D. N. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence may be addressed: Dept. of Dermatology, Yale University School of Medicine, P. O. Box 208059, HRT 610, 15 York St., New Haven, CT 06520-8059. Tel.: 203-785-4352; Fax: 203-785-7637; E-mail: ruth.halaban@yale.edu.

§§ To whom correspondence may be addressed: Dept. of Biochemistry & Molecular Biology, University of Massachusetts, Lederle Graduate Research Tower, 818, Box 34505, Amherst, MA 01003. Tel.: 413-545-0079; Fax: 413-545-3291; dhebert@biochem.umass.edu.

[1] The abbreviations used are: ER, endoplasmic reticulum; Endo H, endoglycosidase H; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; ERGIC, ER-Golgi intermediate compartment; mAb, monoclonal antibody; pHe, extracellular pH; pHi, intracellular pH; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; WGA, wheat germ agglutinin; V-ATPase, vacuolar proton ATPase; PBS, phosphate-buffered saline; DOPA, dihydroxyphenylalanine.

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006

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 alkalinization 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 foreskins in Ham's 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-acetate, 0.1 mM isobutylmethylxanthine, 2.5 nM cholera toxin, 1 μM Na_3VO_4, and 0.1 mM N^6,2′-*O*-dibutyryladenosine-3-AMP (β_2AMP) (TICVA, Sigma) (30).

Human metastatic amelanotic melanoma cells (YUGEN8, 501 mel, YUSIT1, and YUSAC2) (30), were maintained in the Ham's F-10 basal medium. The melanotic Heik178 cells were grown in the Ham's F-10 basal medium supplemented with growth factors (2 ng/ml fibroblast growth factor 2, 10 nM endothelin 1, 10 nM 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 select®-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 mM stock solution in Me_2SO, Sigma), concanamycin A or bafilomycin A1 (20 μM stock solutions dissolved in Me_2SO, both from Calbiochem), pepstatin (10 mg/ml stock solution), or leupeptin (20 mg/ml stock solution, Sigma) using Me_2SO 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 (lectin 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 T311 (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-actin rabbit polyclonal antibodies (Sigma).

Tyrosinase Activity—Tyrosinase assays were performed with L-[3,5-^{3}H]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-^{3}H]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 duplicates, 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% CO_2 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% CO_2 to allow complete hydrolysis of the dye ester. The plates were then mounted on the microscope stage, and pH was monitored at 37 °C under humidified air containing 5% CO_2. 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 [^{35}S]Met/Cys (0.7 mCi/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 radioactive 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; an ER marker). The primary antibodies were detected 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 was visualized with an inverted Bio-Rad MRC-600 laser confocal microscope system. 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 Heik178 remain basic (pH 7.4–7.5). Therefore, we evaluated pigmentation and tyrosinase processing in YUGEN8 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. Alkalinization of the pHe had a marked effect as the cells became pigmented during 2 weeks of culture (Fig. 1*A*). An analysis of steady-state tyrosinase levels demonstrated an increase in the abundance of tyrosinase protein (Fig. 1*B*, *lanes 1* and *2*, normalized to actin and total protein concentration). Most striking was the increase in the level of the higher molecular weight mature tyrosinase (Fig. 1*B*, compare *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*-

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006

The Journal of Biological Chemistry
ASBMB
jbc ONLINE

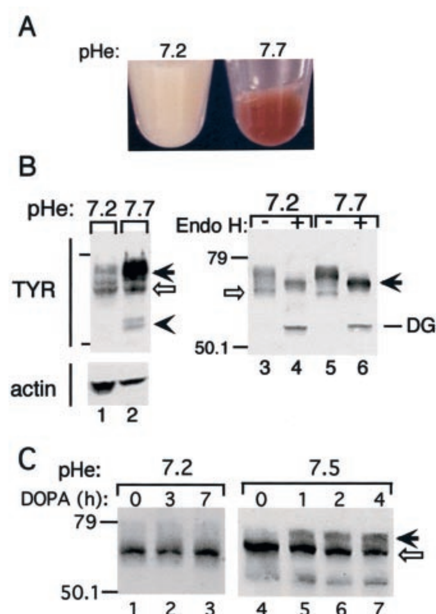

FIG. 1. **Extracellular pH-dependent pigmentation, stabilization, and Golgi processing of tyrosinase.** YUGEN8 melanoma cells were grown in Ham's F-10 medium (pHe ~7.2) or in medium adjusted to pH 7.7 for 2 weeks. *A*, pellets of amelanotic and melanotic cells grown at the indicated pHe. *B*, *lanes 1* and *2* represent anti-tyrosinase Western blot analysis of whole cell lysates prepared from the cell pellets presented in *A*. *Solid* and *empty arrows* and *spearhead* mark mature and immature tyrosinase and proteolytic degradation products, respectively. Protein loading in each well was normalized by subsequent immunoblotting the same membrane with anti-actin antibodies (*actin*). *Lanes 3–6* represent anti-tyrosinase Western blot of proteins bound to WGA beads undigested (−) or digested (+) with Endo H. *Arrow* indicates mature partially Endo H-resistant tyrosinase and the deglycosylated (*DG*) form of the immature glycoform (*empty arrow*). *C*, low pHe-suppressed DOPA-induced Golgi maturation. Western blot analysis of cell extracts derived from YUGEN8 melanoma cells incubated in OptiMEM medium containing 200 μM tyrosine with 50 μM DOPA at pHe 7.2 (*lanes 1–3*) or pHe 7.5 (*lanes 4–7*). *Solid* and *empty arrows* indicate the mature and immature tyrosinase forms as noted above. *Numbers* on the *left-hand side* of Western blot images here and in all other figures indicate molecular mass markers in kDa.

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. 1*B*, *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. 1*B*, *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. 1*B*, *lanes 4* and *6*, *band* marked *DG*). Species of low molecular mass of ~58 kDa (Fig. 1*B*, *lane 2* marked with *spearhead*) also accumulated after incubation in alkaline pHe. This protein band represents the non-glycosylated tyrosinase as shown by its inability to bind WGA (Fig. 1*B*, *lanes 5*) as reported previously (6, 11).

Further evidence that alkaline pHe 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 pHe could suppress the DOPA effect. Indeed maintaining the cells at low pHe hampered the DOPA/tyrosine-induced maturation (Fig. 1*C*, compare *lanes 1–3* with *4–7*), suggesting that long term growth in acidified pH suppresses DOPA activation, tyrosinase maturation, and pigmentation.

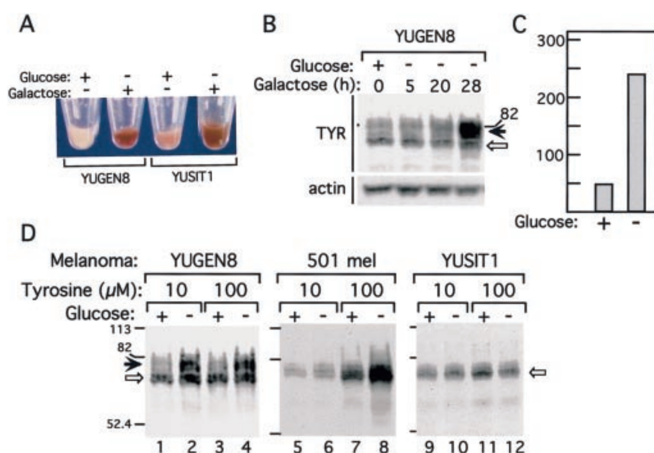

FIG. 2. **Restoration of pigmentation, increased tyrosinase activity, and Golgi maturation by glucose restriction.** Melanoma cells were incubated in glucose-free Dulbecco's modified Eagle's medium with 7% fetal bovine serum plus 1 mM pyruvate supplemented with 25 mM glucose (+) or with 2 mM galactose (−). *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 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 (−) (Fig. 2). The source of energy affected the pH of the external medium because at the end of 3 days of incubation, the pHe was 7.1 and 7.5–7.7, in the glucose-containing and glucose-free (galactose-supplemented) medium, respectively. The levels of pigmentation were also dramatically increased in YUGEN8 and YUSIT1 melanoma cells grown in the glucose-free medium compared with glucose-supplemented medium (Fig. 2*A*, compare + to −). Time-course analysis revealed the accumulation of the 80-kDa tyrosinase glycoform in melanoma cells after 28 h in glucose-free medium (Fig. 2*B*, *TYR*) accompanied by a large increase in tyrosinase activity (Fig. 2*C*). These results demonstrated that glucose metabolism has an impact on tyrosinase activity and processing, probably because of acidification of the extracellular milieu and endomembraneous compartments.

Because tyrosinase maturation has been shown previously to be induced by its activation (11), we further investigated whether the restoration of tyrosinase processing obtained with direct alkalinization of the pHe (Fig. 1) or by restricting glucose

ASBMB
The Journal of Biological Chemistry
jbc ONLINE
Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006

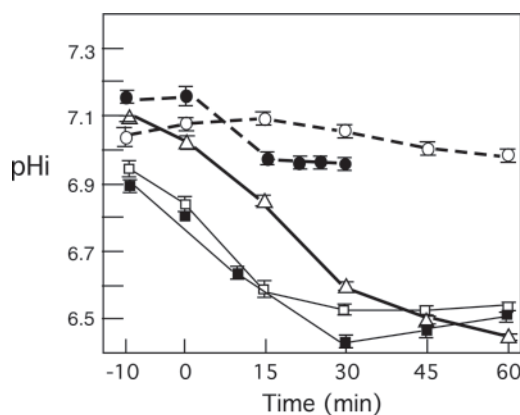

FIG. 3. **Amelanotic melanoma cells maintain high pHi values after acidification.** The pHi of normal human melanocytes (□), amelanotic YUGEN8 (●) and 501 mel (○) melanoma cells, and melanotic Heik178 (■) and MNT1 (△) melanoma cells was measured after exposure to pH 7.0. Measurements were done in growth medium at pH 7.3, 10 min before pH changes (−10 data point) and up to 60 min after a shift to pHe 7.0 (acidification). Reduction in pH during initial recording before time 0 is sometimes seen when the lost CO_2 during transfer to the microscope was not completely reequilibrated. 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 cells, 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. 2*D, 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. 2*D*, 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. 2*D*, *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^-/HCO_3 exchangers are known to be activated in tumor cells to maintain pHi and protect the cells from the acidic extracellular environment (reviewed in Ref. 24). 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 alkalinization 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,[2] 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. 4*A*, 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. 4*A*, *lanes 7*, *8*, *11*, *12*, *15*, *16*, *19*, and *20*). Although high concentrations of tyrosine were not required to elicit maturation (Fig. 4*A*, *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. 4*B*). 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. 4*C*). 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. 4*D*). 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 (43).

Interestingly, despite a marked increase in pigmentation (data not shown), monensin did not affect the SDS-PAGE migration pattern and abundance of tyrosinase (Fig. 4*A*, *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. 5*A*, *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.

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006

ASBMB The Journal of Biological Chemistry jbc ONLINE

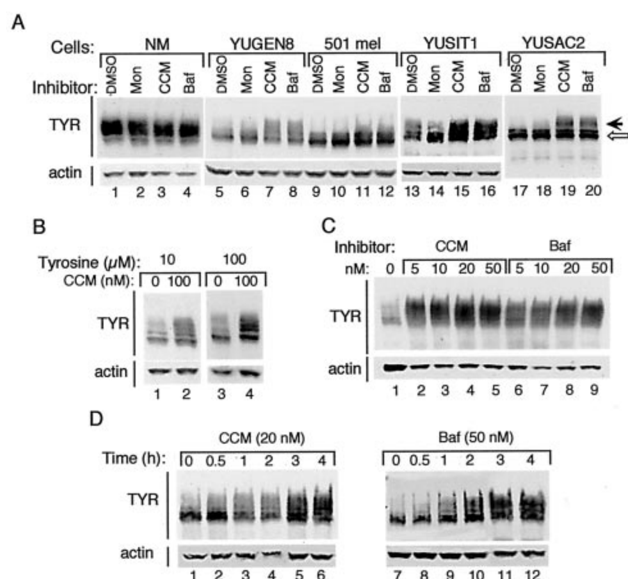

FIG. 4. **Inhibitors of V-ATPase enhance tyrosinase maturation.** *A*, Western blot analyses for tyrosinase (*TYR*) normalized to actin using whole cell lysates derived from normal melanocytes (*NM*) and melanoma cells (YUGEN8, 501 mel, and YUSIT1) or WGA-bound glycoproteins from YUSAC2 melanoma cells. The various cell types were incubated for 4 h before harvest in medium supplemented with the diluent 1 μl/ml Me_2SO (*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.

marked with *empty* and *solid arrows*, respectively). The simultaneous addition of monensin and concanamycin A prevented the concanamycin A induced tyrosinase maturation (Fig. 5*A*, *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. 5*B*, *YUGEN8*, *lanes 1–12*, *17–20*, *bands* marked with *arrow*).

To determine whether tyrosinase stabilization by alkalinization 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. 5*A*, *lanes 13–16*, and *B*, *lanes 13–16*) but not in 501 mel cells (Fig. 5*A*, *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 pigmentation and enhanced maturation of tyrosinase observed after alkalinization or V-ATPase inhibition.

Exit of Tyrosinase from the ER in Response to Intracellular Alkalinization—Confocal immunofluorescence analyses indicated that tyrosinase remained in the ER in melanoma cells

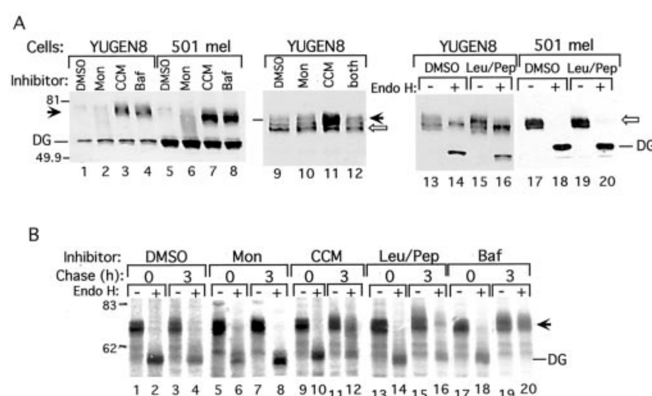

FIG. 5. **Golgi modification of tyrosinase in response to V-ATPase and protease inhibitors.** *A*, steady-state tyrosinase as revealed by anti-tyrosinase Western blotting. Cells were treated with 1 μl/ml Me_2SO (*DMSO*), 20 μM monensin (*Mon*), 20 nM concanamycin A (*CCM*), 50 nM bafilomycin A1 (*Baf*), 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 $[^{35}S]$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.

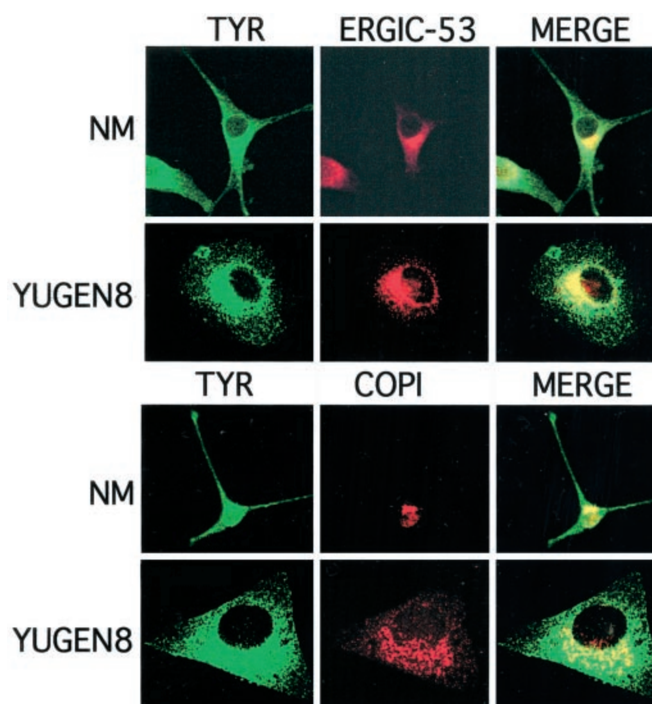

FIG. 6. **Tyrosinase is not present in the ERGIC or *cis*-Golgi in melanoma cells.** Immunofluorescence confocal microscopy images are shown with immunostaining of tyrosinase (*green*) and ERGIC-53 or COPI (*red*) in normal human melanocytes (*NM*) and melanoma cells (*YUGEN8*). The panels on the *right* display merged images. Notice that tyrosinase is spread out in regions that do not include the ERGIC 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*).

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006

ASBMB
The Journal of Biological Chemistry
jbc ONLINE

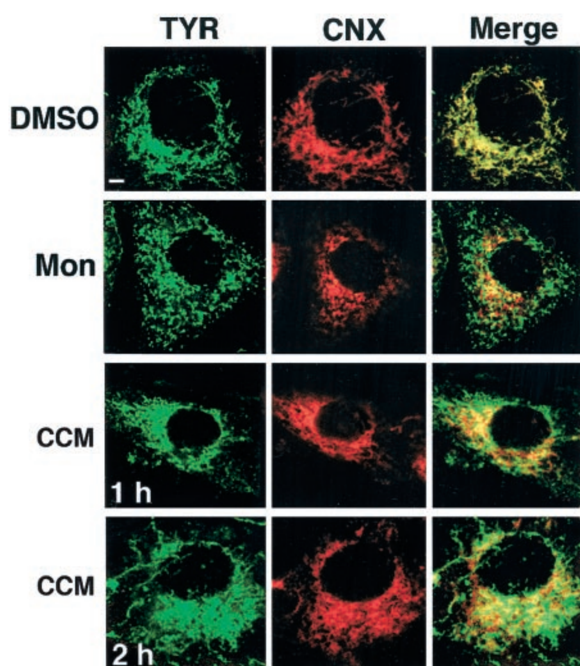

FIG. 7. **Tyrosinase export from the ER is induced by V-ATPase inhibition.** Forced pH changes across vacuolar compartment-induced export of tyrosinase to distal sites. Immunofluorescence confocal microscopy was performed on YUGEN8 melanoma cells grown in Ham's F-10 medium after treatment with 1 μl/ml Me_2SO (*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*).

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, *YUGEN8*). 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, *CCM*, *1 h*). After 2-h treatment, tyrosinase localized in tubules extending from perinuclear compartments across the cell (Fig. 7, *CCM*, *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 alkalinization. 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.

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 alkalinization in a manner dependent on the presence of extracellular tyrosine. Alkalinization 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 pHi 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^-/HCO_3 exchangers are known to be activated in tumor cells in order to maintain pHi, 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 alkalinization 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 vivo*, the *in vitro* activation 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 alkalinization 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

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006
The Journal of Biological Chemistry
ASBMB
jbc ONLINE

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 monensin, 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 monensin acts by dissipating a pH gradient upstream of the concanamycin A-affected 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 (63–66). The P-protein is a 110-kDa melanosomal protein (67) with 12 putative membrane-spanning domains and homology to known transporters (68). In the absence of normal P-protein, there is an imbalance in the intracellular pH of melanosomes, disruption in melanosomal structure, and misrouting of tyrosinase to other sites including the cell membrane (69–71). It was recently suggested that the P-protein acts as a Na^+/H^+ exchanger in the melanosomes (72, 73). The homology to *Escherichia coli* Na^+/H^+ antiporter and the observation that dissipating pH gradients or inhibiting V-ATPase activity in mouse melanocytes carrying the P-mutation restored pigmentation (72) supports this possibility.

We suggest that tyrosinase maturation is particularly vulnerable to pH changes, because of its oxidoreductase activity and its dependence on DOPA for activation. The acidic pH may have inactivated tyrosinase by the protonation of DOPA, the critical cofactor and substrate for tyrosinase (1, 2, 74–76). Unlike tyrosine, DOPA and other catechols can be oxidized to the corresponding quinone by the oxidized form of the enzyme (Cu II state without bound dioxygen), thus reducing the copper atoms in the active site and enabling the generation of the active oxygen-bound form (2, 75, 76). Protonation of DOPA prevents the formation of DOPAquinone, the intermediate required for DOPA regeneration (2, 29, 76). The precise mechanism by which DOPA is regenerated from DOPAquinone is not yet determined, but it is possible that DOPAquinone is reduced to DOPA through the oxidation of critical sulfhydryl groups on tyrosinase, forming the final disulfide bond(s) required to stabilize the protein in its native/active form. In this scenario, the depletion of DOPAquinone leads to the accumulation of misfolded tyrosinase in the ER or the ER-Golgi boundary. The requirement for a tyrosinase reaction product in tyrosinase proper folding may explain the uniqueness of tyrosinase sensitivity to proton changes, because the maturation of the homologous melanocyte-specific glycoprotein, gp75/TRP1, within melanoma cells is unaffected (77).

Our results provide an explanation for the appearance of amelanotic clones in primary and metastatic pigmented tumors in which tyrosinase accumulates in the ER as a result of organelle acidification *in vivo*. It would be of interest to correlate the appearance of amelanotic clones in primary and metastatic pigmented tumors with rates of glucose uptake and the acquisition of drug resistance to further substantiate that down-regulation of tyrosinase is a consequence of these metabolic changes *in vivo*.

Acknowledgments—We thank Drs. L. Old (Memorial Sloan-Kettering Cancer Center, New York, NY) for anti-tyrosinase 3T11 mAb, H.-P. Hauri for the ERGIC-53 antibodies, G. Warren for the rabbit polyclonal anti-β-COPI antibodies, and M. S. Marks for the MNT1 melanoma cells.

REFERENCES

1. Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H. (1949) *J. Biol. Chem.* **178,** 185–195
2. Riley, P. A. (1999) *Cell. Mol. Biol.* **45,** 951–960
3. Kang, X. Q., Kawakami, Y., Elgamil, M., Wang, R. F., Sakaguchi, K., Yannelli, J. R., Appella, E., Rosenberg, S. A., and Robbins, P. F. (1995) *J. Immunol.* **155,** 1343–1348
4. Topalian, S. L., Gonzales, M. I., Parkhurst, M., Li, Y. F., Southwood, S., Sette, A., Rosenberg, S. A., and Robbins, P. F. (1996) *J. Exp. Med.* **183,** 1965–1971
5. Cormier, J. N., Abati, A., Fetsch, P., Hijazi, Y. M., Rosenberg, S. A., Marincola, F. M., and Topalian, S. L. (1998) *J. Immunother.* **21,** 27–31
6. Halaban, R., Cheng, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V., and Hebert, D. N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6210–6215
7. Mosse, C. A., Meadows, L., Luckey, C. J., Kittlesen, D. J., Huczko, E. L., Slingluff, C. L., Shabanowitz, J., Hunt, D. F., and Engelhard, V. H. (1998) *J. Exp. Med.* **187,** 37–48
8. Halaban, R., Svedine, S., Cheng, E., Smicun, Y., Aron, R., and Hebert, D. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5889–5894
9. Berson, J. F., Frank, D. W., Calvo, P. A., Bieler, B. M., and Marks, M. S. (2000) *J. Biol. Chem.* **275,** 12281–12289
10. Toyofuku, K., Wada, I., Spritz, R. A., and Hearing, V. J. (2001) *Biochem. J.* **355,** 259–269
11. Halaban, R., Cheng, E., Svedine, S., Aron, R., and Hebert, D. N. (2001) *J. Biol. Chem.* **276,** 11933–11938
12. Engin, K., Leeper, D. B., Cater, J. R., Thistlethwaite, A. J., Tupchong, L., and McFarlane, J. D. (1995) *Int. J. Hyperthermia* **11,** 211–216
13. Levine, E. A., Holzmayer, T. A., Roninson, I. B., and Das Gupta, T. K. (1993) *J. Surg. Res.* **54,** 621–624
14. Berger, W., Elbling, L., Minai-Pour, M., Vetterlein, M., Pirker, R., Kokoschka, E. M., and Micksche, M. (1994) *Int. J. Cancer* **59,** 717–723
15. Schadendorf, D., Makki, A., Stahr, C., van Dyck, A., Wanner, R., Scheffer, G. L., Flens, M. J., Scheper, R., and Henz, B. M. (1995) *Am. J. Pathol.* **147,** 1545–1552
16. Burd, R., Wachsberger, P. R., Biaglow, J. E., Wahl, M. L., Lee, I., and Leeper, D. B. (2001) *Cancer Res.* **61,** 5630–5635
17. Stubbs, M., McSheehy, P. M., Griffiths, J. R., and Bashford, C. L. (2000) *Mol. Med. Today* **6,** 15–19
18. Dang, C. V., Lewis, B. C., Dolde, C., Dang, G., and Shim, H. (1997) *J. Bioenerg. Biomembr.* **29,** 345–354
19. Warburg, O. (1956) *Science* **123,** 309–314
20. Racker, E. (1983) *Science* **222,** 232
21. Rinne, D., Baum, R. P., Hor, G., and Kaufmann, R. (1998) *Cancer* **82,** 1664–1671
22. Altan, N., Chen, Y., Schindler, M., and Simon, S. M. (1998) *J. Exp. Med.* **187,** 1583–1598
23. Schindler, M., Grabski, S., Hoff, E., and Simon, S. M. (1996) *Biochemistry* **35,** 2811–2817
24. Simon, S. M. (1999) *Drug Discov. Today* **4,** 32–38
25. Martinez-Zaguilan, R., Lynch, R. M., Martinez, G. M., and Gillies, R. J. (1993) *Am. J. Physiol.* **265,** C1015–C1029
26. Martinez-Zaguilan, R., Raghunand, N., Lynch, R. M., Bellamy, W., Martinez, G. M., Rojas, B., Smith, D., Dalton, W. S., and Gillies, R. J. (1999) *Biochem. Pharmacol.* **57,** 1037–1046
27. Yamagata, M., Hasuda, K., Stamato, T., and Tannock, I. F. (1998) *Br. J. Cancer* **77,** 1726–1731
28. Simon, S., Roy, D., and Schindler, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1128–1132
29. Naish-Byfield, S., and Riley, P. A. (1998) *Pigment Cell Res.* **11,** 127–133
30. Halaban, R., Cheng, E., Smicun, Y., and Germino, J. (2000) *J. Exp. Med.* **191,** 1005–1015
31. Cuomo, M., Nicotra, M. R., Apollonj, C., Fraioli, R., Giacomini, P., and Natali, P. G. (1991) *J. Invest. Dermatol.* **96,** 446–451
32. Raposo, G., Tenza, D., Murphy, D. M., Berson, J. F., and Marks, M. S. (2001) *J. Cell Biol.* **152,** 809–824
33. Chen, Y. T., Stockert, E., Tsang, S., Coplan, K. A., and Old, L. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8125–8129
34. Pomerantz, S. H. (1976) *Anal. Biochem.* **75,** 86–90
35. Halaban, R., and Lerner, A. B. (1977) *Exp. Cell Res.* **108,** 119–125
36. Halaban, R., Moellmann, G., Tamura, A., Kwon, B. S., Kuklinska, E., Pomerantz, S. H., and Lerner, A. B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7241–7245
37. Owen, C. S., Pooler, P. M., Wahl, M. L., Coss, R. A., and Leeper, D. B. (1997) *J. Cell. Physiol.* **173,** 397–405
38. Wahl, M. L., Pooler, P. M., Briand, P., Leeper, D. B., and Owen, C. S. (2000) *J. Cell. Physiol.* **183,** 373–380
39. Újvári, A., Aron, R., Eisenhaure, T., Cheng, E., Smicun, Y., Halaban, R., and Hebert, D. N. (2001) *J. Biol. Chem.* **276,** 5924–5931
40. Gillies, R. J., Martinez-Zaguilan, R., Martinez, G. M., Serrano, R., and Perona, R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7414–7418

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006

The Journal of Biological Chemistry

41. Martinez-Zaguilan, R., and Gillies, R. J. (1992) *Ann. N. Y. Acad. Sci.* **671,** 478–480
42. Maxfield, F. R. (1982) *J. Cell Biol.* **95,** 676–681
43. Dröse, S., and Altendorf, K. (1997) *J. Exp. Biol.* **200,** 1–8
44. Fuller, B. B., Spaulding, D. T., and Smith, D. R. (2001) *Exp. Cell Res.* **262,** 197–208
45. Ancans, J., Tobin, D. J., Hoogduijn, M. J., Smit, N. P., Wakamatsu, K., and Thody, A. J. (2001) *Exp. Cell Res.* **268,** 26–35
46. Mellman, I., Fuchs, R., and Helenius, A. (1986) *Annu. Rev. Biochem.* **55,** 663–700
47. Wu, M. M., Llopis, J., Adams, S., McCaffery, J. M., Kulomaa, M. S., Machen, T. E., Moore, H. P., and Tsien, R. Y. (2000) *Chem. Biol.* **7,** 197–209
48. Tartakoff, A. M. (1983) *Cell* **32,** 1026–1028
49. Tartakoff, A. M., and Vassalli, P. (1977) *J. Exp. Med.* **146,** 1332–1345
50. Strous, G. J., and Lodish, H. F. (1980) *Cell* **22,** 709–717
51. Forgac, M. (1999) *J. Biol. Chem.* **274,** 12951–12954
52. Nelson, N., and Harvey, W. R. (1999) *Physiol. Rev.* **79,** 361–385
53. Finbow, M. E., and Harrison, M. A. (1997) *Biochem. J.* **324,** 697–712
54. Stevens, T. H., and Forgac, M. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 779–808
55. Palokangas, H., Ying, M., Vaananen, K., and Saraste, J. (1998) *Mol. Biol. Cell* **9,** 3561–3578
56. Kim, J. H., Johannes, L., Goud, B., Antony, C., Lingwood, C. A., Daneman, R., and Grinstein, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2997–3002
57. Wu, M. M., Llopis, J., Adams, S. R., McCaffery, J. M., Teter, K., Kulomaa, M. S., Machen, T. E., Moore, H. P., and Tsien, R. Y. (2000) *Methods Enzymol.* **327,** 546–564
58. Schmidt, W. K., and Moore, H. P. (1995) *Mol. Biol. Cell* **6,** 1271–1285
59. Ellgaard, L., Molinari, M., and Helenius, A. (1999) *Science* **286,** 1882–1888
60. Hammond, C., and Helenius, A. (1994) *J. Cell Biol.* **126,** 41–52
61. Raposo, G., Vansanten, H. M., Leijendekker, R., Geuze, H. J., and Ploegh, H. L. (1995) *J. Cell Biol.* **131,** 1403–1419
62. Yamamoto, K., Fujii, R., Toyofuku, Y., Saito, T., Koseki, H., Hsu, V. W., and Aoe, T. (2001) *EMBO J.* **20,** 3082–3091
63. Gardner, J. M., Nakatsu, Y., Gondo, Y., Lee, S., Lyon, M. F., King, R. A., and Brilliant, M. H. (1992) *Science* **257,** 1121–1124
64. Rinchik, E. M., Bultman, S. J., Horsthemke, B., Lee, S. T., Strunk, K. M., Spritz, R. A., Avidano, K. M., Jong, M. T., and Nicholls, R. D. (1993) *Nature* **361,** 72–76
65. Lee, S. T., Nicholls, R. D., Bundey, S., Laxova, R., Musarella, M., and Spritz, R. A. (1994) *N. Engl. J. Med.* **330,** 529–534
66. Oetting, W. S., Gardner, J. M., Fryer, J. P., Ching, A., Durham-Pierre, D., King, R. A., and Brilliant, M. H. (1998) *Hum. Mutat.* **12,** 434
67. Rosemblat, S., Durham-Pierre, D., Gardner, J. M., Nakatsu, Y., Brilliant, M. H., and Orlow, S. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12071–12075
68. Brilliant, M. H., Gondo, Y., and Eicher, E. M. (1991) *Science* **252,** 566–569
69. Puri, N., Gardner, J. M., and Brilliant, M. H. (2000) *J. Invest. Dermatol.* **115,** 607–613
70. Orlow, S. J., and Brilliant, M. H. (1999) *Exp. Eye Res.* **68,** 147–154
71. Potterf, S. B., Furumura, M., Sviderskaya, E. V., Santis, C., Bennett, D. C., and Hearing, V. J. (1998) *Exp. Cell Res.* **244,** 319–326
72. Ancans, J., Hoogduijn, M. J., and Thody, A. J. (2001) *J. Invest. Dermatol.* **117,** 158–159
73. Brilliant, M. H. (2001) *Pigment Cell Res.* **14,** 86–93
74. Raper, H. S. (1928) *Physiol. Rev.* **8,** 245–282
75. Riley, P. A. (1998) in *The Pigmentary System. Physiology and Pathophysiology* (Nordlund, J. J., Boissy, R., Hearing, V. J., King, R. A., and Ortonne, J-P., eds) 1st Ed., pp. 401–421, Oxford University Press, New York., New York
76. Cooksey, C. J., Garratt, P. J., Land, E. J., Pavel, S., Ramsden, C. A., Riley, P. A., and Smit, N. P. (1997) *J. Biol. Chem.* **272,** 26226–26235
77. Vijayasaradhi, S., Xu, Y. Q., Bouchard, B., and Houghton, A. N. (1995) *J. Cell Biol.* **130,** 807–820

The Journal of Biological Chemistry

ASBMB

jbc ONLINE

Downloaded from www.jbc.org at UNIV OF MASSACHUSETTS, Amherst on July 21, 2006