

Proper Folding and Endoplasmic Reticulum to Golgi Transport of Tyrosinase Are Induced by Its Substrates, DOPA and Tyrosine*

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Tyrosinase is essential for pigmentation and is a source of tumor-derived antigenic peptides and cellular immune response. Wild type tyrosinase in melanoma cells and certain albino mutants in untransformed melanocytes are targeted to proteolytic degradation by the 26 S proteasome due to retention of the misfolded protein in the endoplasmic reticulum and its subsequent retranslocation to the cytosol. Here, we demonstrate that the substrates DOPA and tyrosine induced in melanoma cells a transition of misfolded wild type tyrosinase to the native form that is resistant to proteolysis, competent to exit the endoplasmic reticulum, and able to produce melanin. Because the enzymatic activity of tyrosinase is induced by DOPA, we propose that proper folding of the wild type protein, just like mutant forms, is tightly linked to its catalytic state. Loss of pigmentation, therefore, in tyrosinase-positive melanoma cells is a consequence of tumor-induced metabolic changes that suppress tyrosinase activity and DOPA production within these cells.

Loss of pigmentation in human melanoma cells is common in advanced and metastatic lesions due to suppression of melanocyte-specific proteins. Down-regulation at the transcriptional level is likely to involve inactivation of microphthalmia, the transcription factor controlling pigment-specific genes such as tyrosinase, the key enzyme in melanin synthesis (1, 2). However, post-transcriptional processes cause the amelanotic phenotype of melanoma cells expressing wild type tyrosinase. In these cells tyrosinase is synthesized at levels similar to those of pigmented normal melanocytes but is retained in the endoplasmic reticulum (ER)¹ as an early 70-kDa glycoform that is subsequently degraded by the proteasome (3). Retention of tyrosinase in the ER is also characteristic of mutant tyrosinase proteins associated with oculocutaneous albinism type 1 (4, 5).

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¹ The abbreviations used are: ER, endoplasmic reticulum; TYR, tyrosinase; WGA, wheat germ agglutinin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; IBMX, 3-isobutyl-1-methyl xanthine; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Endo H, endoglycosidase H; TH, tyrosine hydroxylase; mAb, monoclonal antibody.

Individuals with this recessively inherited condition have a normal number of melanocytes in the skin that are defective in melanin production. Thus, in melanoma cells, wild type tyrosinase behaves like a mutant protein in that it is not processed to the 80-kDa mature form, and its level in the melanosomes, the site of melanin synthesis, is not sufficient to induce pigmentation. These observations explain not only the loss of pigmentation but also suggest a mechanism by which tyrosinase peptides are produced for antigen presentation at the cell surface (see for example Refs. 6 and 7).

Tyrosinase (monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1) is a copper-containing enzyme that catalyzes the oxidation of tyrosine and DOPA to DOPAquinone, the rate-limiting reactions in melanin synthesis (8). Because the catalytic activity of tyrosinase is dependent on DOPA (8–10), we explored the possibility that DOPA also elicits a conformational change favorable for exit of tyrosinase from the ER to the Golgi. Here we show that the release of wild type tyrosinase from the ER was enhanced in melanoma cells by treatment with the cofactor DOPA and the substrate tyrosine. DOPA/tyrosine promoted the accumulation of proteolytically resistant and Golgi-processed tyrosinase, as confirmed by biochemical analyses and immunofluorescence microscopy.

EXPERIMENTAL PROCEDURES

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 Life Technologies, Inc.), termed basal medium, that was further enriched with several ingredients required for normal melanocyte proliferation. They included 85 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 0.1 mM 3-isobutyl-1-methyl xanthine (IBMX), 2.5 nM cholera toxin, 1 μ M Na₂VO₄, and 0.1 mM dbcAMP, all from Sigma (11) designated TICVA. The highly pigmented state of normal melanocytes was stable regardless of passage number or changes in the composition of the external growth factors.

Human metastatic melanoma cells YUGEN8, 501 mel, 888 mel, YUSAC2, and YUSIT1 (all expressing wild type tyrosinase) were maintained in Ham's F-10 basal medium as described (11). Experiments were performed with the basal medium and unmodified OptiMEM (Life Technologies, Inc.) plus 2% fetal bovine serum or tyrosine-free minimal essential medium or RPMI medium. Experimental medium was supplemented with 50 μ M freshly prepared DOPA (stock solution 1 mM in PBS) or tyrosine (Life Technologies, Inc.; at the indicated concentration) and adjusted to pH 7.4–7.7 prior to use. The concentration of tyrosine in the unmodified Ham's F-10 and OptiMEM media was 10 and 200 μ M, respectively. To test the effect of tyrosine elimination, cells were starved in tyrosine-free medium for 2–4 h prior to any further manipulations. The effect of external tyrosinase activity was explored by adding cell extract of normal human melanocytes prepared in a solution containing 0.5% CHAPS, 50 mM HEPES, and 200 mM NaCl, pH 7.5. Tyrosinase activity in the melanocyte cell extract was determined as described below.

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, Indianapolis, IN) was used to lyse cells and to wash bead-bound precipitated material as described (3). Immunoblotting analyses of whole cell lysates (40 μg protein/lane, as measured by the Bio-Rad protein assay reagent), or affinity purified glycoproteins using wheat germ agglutinin (WGA) bound to beads (lectin from *triticum vulgare*; Sigma) were performed following standard procedures or the manufacturer's instructions. Tyrosinase was detected with mouse monoclonal antibody T311 (12), and equal protein loading was verified by staining the gels with Coomassie Brilliant Blue after transfer of the proteins to membranes and by subsequent immunoblotting with anti-actin antibodies. Densities of tyrosinase bands on the x-ray films were determined using a NIH image analyzer, selecting all visible bands, as described in the manual.

Carbohydrate Cleavage—Cell lysates (200 μg of protein in ~ 100 μl of CHAPS lysis buffer) were incubated with 50–70- μl slurry of WGA bound to beads and shaking for 2 h at 8 $^{\circ}\text{C}$. Bead-bound proteins were hydrolyzed with endoglycosidase H (Endo H) according to the manufacturer's instruction (Roche Molecular Biochemicals). Reactions were stopped with sample buffer at 95 $^{\circ}\text{C}$ for 5 min, and proteins were subjected to Western blotting as above.

Immunofluorescence Microscopy—Melanoma cells were grown on chamber slides in unmodified Ham's F-10 medium at pH 7.6. Cells were incubated with and without 50 μM DOPA in Ham's F-10 medium for 4 h. Attached 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 polyclonal antibodies against tyrosinase (goat C-19; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or calnexin (rabbit; StressGen Biotechnologies Corp., Victoria, BC, Canada) to localize the ER and then with fluorescein anti-goat conjugates (Santa Cruz Biotechnology Inc.) or rhodamine anti-rabbit conjugates (Molecular Probes, Eugene, OR), all diluted 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.

Trypsin Digestion—Whole cell lysates (4 μg protein/ μl in 2% CHAPS lysis buffer without protease inhibitors) were digested with trypsin (TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone)-treated) at the indicated concentration at 27 $^{\circ}\text{C}$ for 10 or 15 min. Digestion was stopped by the addition of an equal volume of 3 \times SDS sample buffer and heating for 1 min at 95 $^{\circ}\text{C}$. Reaction products (40 μg /lane) were subjected to Western analysis with anti-tyrosinase mAb followed by immunoblotting with polyclonal antibodies against actin (Sigma) as a control.

Metabolic Labeling—Pulse-chase experiments were performed as described (4). Briefly, cells were pulse-labeled (15 min) with [^{35}S]Met/Cys (0.7 mCi/ml; EasyTag, PerkinElmer Life Sciences) in methionine/cysteine-free RPMI medium (200 μM tyrosine; Life Technologies, Inc.) and either collected immediately or after 4 h of chase incubation with nonradioactive medium. DOPA (50 μM) was added to half of the samples during the pulse, chase, or both incubation times. Cell extracts ($2\text{--}10 \times 10^7$ cpm in trichloroacetic acid-precipitable material) were treated with Zysorbin (fixed and killed *Staphylococcus aureus*; Zymed Laboratories Inc., San Francisco, CA) to remove nonspecific binding proteins and then subjected to immunoprecipitation with mouse monoclonal T311 anti-tyrosinase mAb. Following extensive washing with radioimmune precipitation buffer, half of the precipitated products were digested with Endo H overnight followed by SDS polyacrylamide gel electrophoresis fractionation and autoradiography. Densities of radioactive tyrosinase bands on the x-ray films were determined using a Molecular Dynamics PhosphorImager.

Tyrosinase Activity—Total cell lysates were prepared in 2% CHAPS buffer plus protease inhibitors as above, and tyrosinase assays were performed with L-tyrosine-[3,5- ^3H] (PerkinElmer Life Sciences) as a substrate (13–15). Briefly, reaction mixtures (200- μl final volume), contained 50–150 μg of cell extract protein, 50 μM L-tyrosine, and 50 μM L-DOPA (in triplicates or duplicates, as indicated) and 1 μCi /assay L-tyrosine-[3,5- ^3H]. After 15–120 min (as indicated) of incubation at 37 $^{\circ}\text{C}$, reactions were stopped with 200 μl of charcoal slurry, passed through 350- μl packed Dowex columns, 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 of tyrosine in 1 min. Standard errors were in the range of 15% of total counts.

RESULTS

DOPA-induced Tyrosinase Maturation—The amelanotic YUGEN8 human melanoma cells that originated from a pigmented metastatic tumor were first tested for the effect of

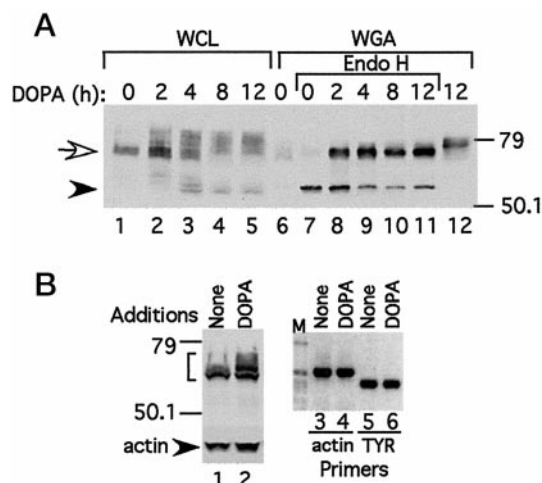


FIG. 1. DOPA-induced Golgi processing and increased tyrosinase levels. A, YUGEN8 melanoma cells were incubated for increasing duration in unmodified OptiMEM medium (5 ml of 200 μM tyrosine), in the presence or absence of 50 μM DOPA. The pH value was maintained at 7.4. Western blotting was performed with whole cell lysates (WCL) or with WGA precipitated proteins that were incubated overnight with or without Endo H. The open and closed arrows designate the 70-kDa ER form and the 60-kDa deglycosylated form of tyrosinase, respectively. B, tyrosinase processing in 501 mel cells in response to DOPA. Cells were incubated with 50 μM DOPA in OptiMEM medium and harvested for Western blot analysis with anti-tyrosinase mAb followed by blotting with anti-actin (lanes 1 and 2). Tyrosinase is marked with a bracket. Alternatively, the same batch of treated cells were harvested and processed for mRNA analysis using quantitative RT polymerase chain reaction and primers for human actin (lanes 3 and 4) or human TYR (lanes 5 and 6). M, size markers 1 and 0.5 kb.

DOPA, because they displayed unstable pigmentation and variability in the levels of ER- and Golgi-processed tyrosinase proteins (3). The cells were incubated in medium supplemented with DOPA for increasing periods of time. Visual examination of the harvested and sedimented cell pellets revealed increased pigmentation after 4 h of DOPA treatment (data not shown).

Western blotting with anti-tyrosinase antibodies of melanoma whole cell lysates, or affinity fractionated glycoproteins digested with Endo H, confirmed previous observations (3). Under conditions that allowed tyrosinase to become fully mature in normal human melanocytes (Ref. 3; see also Fig. 3B, lane 9), the steady-state tyrosinase in the amelanotic YUGEN8 melanoma cells was mainly a distinct ~ 70 -kDa species sensitive to Endo H digestion, indicative of an immature ER glycoform (Fig. 1A, lanes 1 and 7, empty arrow). However, within 2 h of DOPA treatment, mature Endo H-resistant glycoforms accumulated (Fig. 1A, lanes 2 and 8). Most of the enzyme was fully processed and Endo H-resistant after 8 h of treatment (Fig. 1A, lanes 4, 5, 10, and 11). In addition, DOPA caused an increase in the abundance of the 70-kDa species, as well as the 6D-kDa forms, distinctly visible after 2 and 4 h of incubation, respectively (Fig. 1A, lanes 2 and 3, bands marked with empty arrow and arrowhead, respectively). Tyrosinase of 60 kDa was previously identified as the deglycosylated protein that accumulated after treatment with the proteasome inhibitors MG132 or lactacystin but not with the lysosomal inhibitors E64 (*L*-trans-epoxysuccinic acid) and NH_4Cl (3). Therefore, DOPA stabilized the ER glycoform of tyrosinase, as well as protein that had been diverted from the ER to the cytoplasm for degradation.

DOPA-induced tyrosinase maturation and stability was not restricted to YUGEN8 melanoma cells but was also observed in other cell lines originating from pigmented or nonpigmented metastatic tumors (16) (Fig. 1B, compare lane 1 to lane 2, and data not shown). Quantitative RT polymerase chain reaction

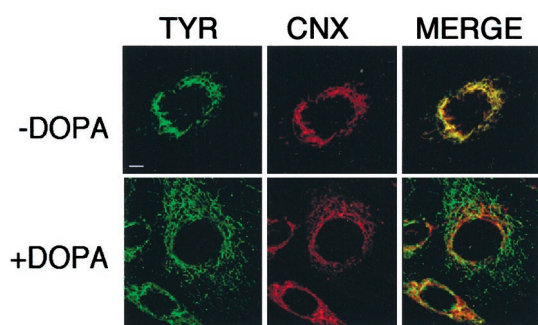


FIG. 2. DOPA induces ER export of tyrosinase to distal sites. Immunofluorescence confocal microscopy of YUGEN8 melanoma cells grown in Ham's F-10 medium before and after treatment with 50 μM DOPA for 4 h is shown. The *left panels* stained with green fluorescein represent TYR detected with polyclonal anti-tyrosinase antibodies. The *middle panels* stained with red rhodamine show the ER resident calnexin (CNX), and the *right panels* display merged images. Tyrosinase colocalization with calnexin is indicated in yellow in the merge. The bars represent 1 μm .

analysis of tyrosinase mRNA showed no increase in message levels (Fig. 1B, compare lane 5 to lane 6), suggesting that the changes occurred post-transcriptionally. Taken together, the increased abundance of tyrosinase species of 70 and 60 kDa and the accumulation of Endo H-resistant glycoforms indicated that DOPA induced a conformational change that conferred resistance to endogenous proteolytic degradation and competency to exit from the ER to the Golgi.

DOPA Treatment Enhanced Tyrosinase Export from the ER to Distal Sites—Subcellular localization by indirect confocal immunofluorescent microscopy confirmed the biochemical analyses. Tyrosinase in amelanotic YUGEN8 melanoma cells was displayed in a perinuclear reticular pattern coincident with the ER marker calnexin (Fig. 2, panels marked -DOPA, compare TYR and CNX to Merge). In contrast, after 4 h of incubation with DOPA, tyrosinase was distributed outside of the ER and Golgi, as observed by the abundant appearance of spots at distal sites that did not merge with calnexin staining (Fig. 2, panels marked +DOPA, compare TYR and CNX to Merge). Therefore, DOPA treatment permitted tyrosinase to become transport-competent.

Tyrosine Is Required for DOPA-enhanced Maturation—As the experiments described above were performed in Ham's F-10 or OptiMEM medium containing tyrosine (10 and 200 μM , respectively), DOPA supplementation may have served to enhance tyrosinase maturation in cooperation with tyrosine. Indeed, the requirement for substrate in this process was confirmed by exclusion of tyrosine from the medium. There was no induction of DOPA-mediated tyrosinase maturation in tyrosine-free minimal essential medium (Fig. 3A, compare lanes 1 and 2), indicating that the substrate and cofactor of tyrosinase are both critical for inducing export-competent tyrosinase species.

We then explored the possibility that tyrosine by itself can induce maturation, because DOPA is generated by an autocatalytic process during the initial phase of the tyrosinase reaction (8–10). In these experiments, accumulation of endogenously produced DOPA was favored by supplementing the medium with excess tyrosine and maintaining high ratio of melanoma cells to medium (such as $\sim 1.5 \times 10^5$ cells/cm²/ml). The results showed that enriching the medium with additional tyrosine to a final concentration of 400 μM was sufficient to induce maturation of YUGEN8 tyrosinase within 2 h (Fig. 3A, compare lane 3 to lanes 5 and 6). Accumulation of tyrosinase reaction products sufficient to stabilize tyrosinase and induce maturation was dependent on the concentration of extracellular tyrosine,

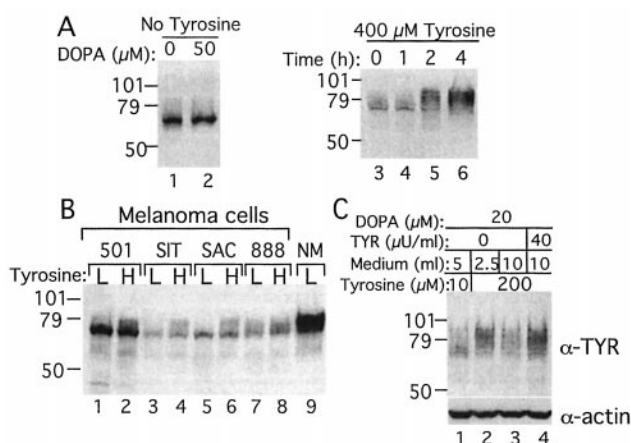


FIG. 3. Tyrosine is required to induce maturation of tyrosinase. Western blots with anti-tyrosinase mAb T311 of whole cell lysates derived from melanoma cells seeded at high densities ($\sim 1 \times 10^6$ cells) in 25-cm² flasks and incubated for short periods in medium manipulated to affect endogenous tyrosinase activity, compared with normal melanocytes, are shown. A, YUGEN8 melanoma cells were deprived of tyrosine for 4 h. Cultures were incubated with fresh tyrosine-free medium without or with DOPA for an additional 4 h (lanes 1 and 2, respectively). Alternatively, cultures of YUGEN8 melanoma cells were incubated in 2.5 ml of unmodified OptiMEM medium (200 μM tyrosine; lane 3) or medium that was supplemented to 400 μM tyrosine final concentration and harvested at different time intervals (lanes 3–6). B, melanoma cells were incubated in 3 ml of unmodified (200 μM tyrosine; lanes 1, 3, 5, and 7) or modified (400 μM tyrosine; lanes 2, 4, 6, and 8) OptiMEM medium for 16 h. Tyrosinase from normal human melanocytes (NM) grown in Ham's F-10 medium (10 μM tyrosine) is shown in lane 9. SIT, YUSIT1; SAC, YUSAC2; L, 200 μM tyrosine; H, 400 μM tyrosine. C, maturation of tyrosinase is manipulated by the concentrations of accumulated extracellular tyrosinase reaction products. YUGEN8 melanoma cells were incubated overnight in the presence of DOPA (20 μM) in 5 ml of unmodified Ham's F-10 medium (lane 1), 2.5 or 10 ml of unmodified OptiMEM medium (lanes 2 and 3, respectively), or 10 ml of OptiMEM medium supplemented with cell extract (60 $\mu\text{g/ml}$) from normal human melanocytes containing 40 microunits/ml TYR activity (lane 4). The membrane was probed consecutively first with anti-tyrosinase (α -TYR) and then with anti-actin polyclonal rabbit antibodies (α -actin).

the level of residual tyrosinase within the melanoma cells, the density of the cells, the volume of the medium, and the duration of the incubation (Fig. 3A, lanes 3–6; B, lanes 1–8; C, lanes 1–3, and data not shown).

The likely enzymatic source of intracellular DOPA in the tyrosine-supplemented medium is tyrosinase and not the dopaminergic enzyme tyrosine hydroxylase (TH). These melanoma cells did not express TH mRNA (tested by RT polymerase chain reaction with TH-specific primers; data not shown), and endogenous TH protein was not detected by Western blotting with anti-TH antibodies (sc-7847; Santa Cruz Biotechnology Inc.; data not shown). Furthermore, ectopic expression of L-DOPA-producing TH rendered constitutively active by truncation of the N-terminal regulatory domain (termed tHTH; see Ref. 17) did not induce any changes in tyrosinase processing in 501 mel amelanotic melanoma cells (data not shown). In contrast, the effect of DOPA and tyrosine on maturation was enhanced by exogenous tyrosinase from normal human melanocytes (40 microunits/ml) supplied to the medium as whole cell extract (Fig. 3C, compare lanes 3 and 4). Altogether, these results showed that aberrant ER retention of tyrosinase in melanoma cells could be corrected by the concerted effect of the cofactor DOPA and the substrate tyrosine.

Stabilization of Tyrosinase—DOPA and tyrosine enhanced the formation of tyrosinase that was more resistant to endogenous proteolytic degradation, as seen by accumulation of the 70-kDa early glycoforms and the apparent deglycosylated 60-

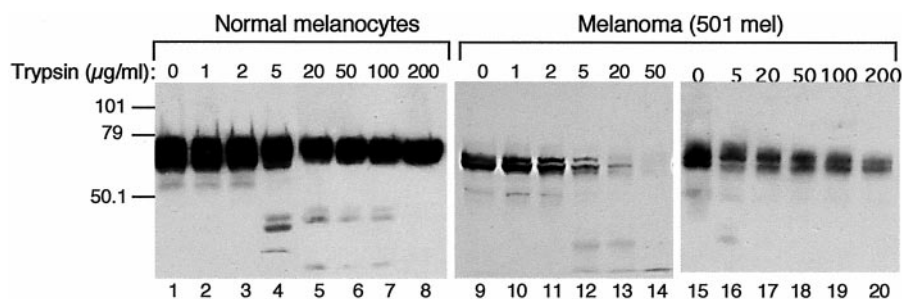


FIG. 4. **Melanoma ER-70-kDa tyrosinase is sensitive to trypsin digestion.** Tyrosinase trypsin digests of cell extracts from normal melanocytes and 501 mel cells grown continuously in unmodified Ham's F-10 medium (10 μM tyrosine; lanes 1–14) and 501 mel cells incubated in modified OptiMEM medium (400 μM tyrosine) for 5 h (lanes 15–20) are shown. Trypsin digestion was for 10 (lanes 1–4 and 9–20) or 15 min (lanes 5–8). Digestion products were subjected to Western blotting with T311 tyrosinase mAb.

kDa species (Fig. 1A, lanes 2 and 3). Therefore, we further explored structural differences between the 70-kDa and fully processed protein by probing their resistance to trypsin digestion using tyrosinase from normal human melanocytes as a control. The results showed that the Golgi-processed enzyme produced in normal human melanocytes was resistant to up to 200 μg of trypsin, whereas the faster migrating 70-kDa ER form was trypsin-sensitive (Fig. 4, lanes 1–8). In melanoma cells that solely accumulated the 70-kDa protein, TYR was highly sensitive to proteolytic digestion as it was completely digested by 50 μg of trypsin in 10 min (Fig. 4, lanes 9–14). Incubation of 501 mel melanoma cells with 400 μM tyrosine for 5 h induced the accumulation of processed tyrosinase protein that was trypsin-resistant up to 200 $\mu\text{g}/\text{ml}$ of trypsin (Fig. 4, lanes 15–20). In contrast, high levels of tyrosine in the medium did not confer trypsin resistance to actin (data not shown). These results suggest that the ER-retained tyrosinase is a trypsin-sensitive conformer, compared with the trypsin-resistant Golgi-processed species whose production was enhanced by the presence of high concentrations of tyrosine in the medium.

Pulse-chase metabolic labeling experiments were carried out to determine the stage at which DOPA stabilized the ER-retained tyrosinase. As shown in Fig. 5, newly synthesized tyrosinase remained an ER-retained and Endo H-sensitive conformer in the absence of DOPA (Fig. 5, lanes 1, 2, 5, and 6). The addition of DOPA during the 4-h chase period was sufficient to stabilize the protein into an exit-competent conformation and induced maturation into an Endo-H resistant form (Fig. 5, lanes 7 and 8). Including DOPA during the pulse and chase periods further enhanced its stability and maturation (Fig. 5, lanes 9 and 10). These results indicated that DOPA stabilization of tyrosinase occurred both co- and post-translationally.

Low Tyrosinase Activity as the Cause for DOPA Depletion—The data suggested that DOPA is in low abundance in melanoma cells probably because of inactive tyrosinase. Because normal melanocytes always possessed Golgi-processed tyrosinase even in the low tyrosine Ham's F-10 medium, we tested whether the mitogenic ingredients (TICVA) added to maintain growth and known to stimulate intracellular levels of cAMP (cholera toxin and IBMX), or protein kinase C (the phorbol ester TPA), can also promote maturation. However, no changes in tyrosinase processing or activity were observed in the melanoma cells in response to TPA, IBMX, or TICVA (data not shown). Conversely, normal melanocytes expressed normal tyrosinase glycoforms and remained highly pigmented when grown in the presence of peptide growth factors, such as basic fibroblast growth factor, hepatocyte growth factor, and endothelin (without TICVA) (18, 19).

Therefore, we tested whether the ER form of tyrosinase from melanoma cells can be activated by DOPA and tyrosine after

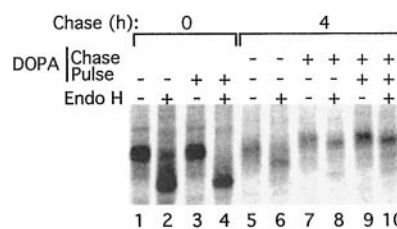


FIG. 5. **DOPA-induced proper folding of newly synthesized and pre-existing tyrosinase early glycoforms.** Melanoma cells YUGEN8 were pulsed with [^{35}S]Met/Cys in RPMI medium for 15 min in the absence or presence of 50 μM DOPA and harvested immediately or after a 4-h chase in nonradioactive RPMI media in the absence or presence of 50 μM DOPA. The RPMI medium contained 200 μM tyrosine. After tyrosinase precipitation with anti-tyrosinase antibodies, half of the bound immunoprecipitates were subjected to Endo H digestion.

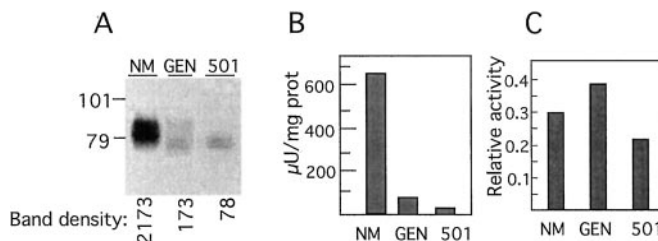


FIG. 6. **Tyrosinase activity in normal versus malignant melanocytes.** A, immunoblot (T311 mAb) of whole cell lysates showing tyrosinase species present at the time the extracts from normal melanocytes (NM), YUGEN8 (GEN), and 501 mel (501) melanoma cells were used to measure DOPA-catalyzed tyrosinase activity. The number under each lane represents arbitrary units of band density of all bands within each lane assessed with an NIH image analyzer. B, tyrosinase activity. A histogram showing tyrosinase activity measured in the presence of DOPA expressed as microunits/mg total protein is shown. Data are averages of triplicate values. Each tyrosinase assay was repeated at least once with similar results. C, histogram showing tyrosinase activity of each cell type (B) normalized to the respective band density displayed in A.

cell lysis. Tyrosinase activity in whole cell lysates from normal melanocytes that represented largely the mature form and from YUGEN8 and 501 mel melanoma cells expressing low levels of proteins mostly of the immature Endo H-sensitive forms was measured in the presence of DOPA (Fig. 6A; see also Ref. 3). Compared with normal melanocytes, extracts from melanoma cells possessed low levels but nevertheless measurable tyrosinase activity (Fig. 6B). This activity is likely a result of the post-lysis activation of the immature protein, because standardizing the activity to tyrosinase band density displayed by the Western blot (Fig. 6A, numbers indicated on the bottom) eliminated the differences between the three samples (Fig. 6C). Thus, DOPA in the *in vitro* assay clearly activated not only fully matured but also immature tyrosinase species.

DISCUSSION

Malignant transformation of normal melanocytes to melanoma cells is associated with down-regulation of pigmentation. In previous studies, we showed that tumor-specific retention of tyrosinase in the ER is the cause of the amelanotic phenotype in tyrosinase-positive melanoma cells (3). Thus, wild type tyrosinase in these cells displays a phenotype similar to inactive mutant tyrosinase proteins common in oculocutaneous albinism type 1 (4, 5). In this report, we provide evidence that DOPA and tyrosine, the cofactor and substrates of tyrosinase, are required for wild type tyrosinase maturation in melanoma cells. DOPA and tyrosine stabilize the ER-retained protein and permit its transport through the secretory pathway in these amelanotic melanoma cells.

The two lectin ER molecular chaperones, calnexin and calreticulin, appear to be responsible for the ER retention of incompletely or improperly folded tyrosinase (3, 4, 20, 21). These chaperones bind and retain proteins possessing monoglycosylated glycans (22, 23). The retention of a protein is the result of continued reglycosylation of the protein by the UDP-glucose:glycoprotein glucosyltransferase, an ER sensor that reglycosylates glycoproteins containing misfolded or mobile regions (24). Therefore, DOPA/tyrosine binding must stabilize tyrosinase so that it is no longer a substrate for reglycosylation and is therefore subsequently released from the ER.

DOPA/tyrosine can be added to the growing list of chemical chaperones that stabilize target proteins, including otherwise inactive mutants. For example, drug substrates or modulators allowed mutant forms of the human ATP-dependent drug pump, P-glycoprotein, to attain an ER export-competent configuration (25, 26). In this case, core-glycosylated, trypsin-sensitive, and ER-retained mutants of human P-glycoprotein were converted to the mature trypsin-resistant form by synthesis in the presence of a drug substrate. Likewise, nonpeptidic antagonists increased cell surface expression and rescued the function of mutant V2 vasopressin receptor (27). In another case, small synthetic molecules promoted the stability of wild type p53 and allowed mutant p53 to maintain an active conformation (28). Although the ER-retention defect of mouse albino mutant tyrosinase C85S (4, 5) could not be corrected by incubating the melanocytes with DOPA/tyrosine (data not shown), it is possible that other less severe albino mutations, such as those causing temperature-sensitivity, may be rescued by DOPA/tyrosine treatment.

For over 70 years it has been known that DOPA activates tyrosinase (8, 29). However, its mechanism of activation has been elucidated only recently by Riley and colleagues (9, 30, 31). These investigators demonstrated that, unlike tyrosine, DOPA (and other catechols) can be oxidized to the corresponding quinone by tyrosinase with oxidized copper atoms (without bound dioxygen), thus reducing the copper atoms in the active site and enabling the generation of the active, oxygen-bound form. It is tempting to speculate that only the oxygen-bound active enzyme is competent to exit the ER, and conditions that block its formation, such as mutations common in albinism or tumor-induced metabolic changes, can result in ER retention and subsequent degradation.

Promotion of tyrosinase folding required the specific interaction with DOPA. Glycerol, a chemical chaperone shown to stabilize immature, core-glycosylated, mutant cystic fibrosis transmembrane conductance regulator molecules that are normally degraded rapidly (32), was not effective in inducing tyrosinase maturation.² The likely cellular source of DOPA is tyrosinase. Incubation of melanoma cells in high levels of tyro-

sine, in the absence of supplemented DOPA, also caused release from the ER to the Golgi. We suggest that autocatalytic activation by tyrosine and subsequent build up of DOPA induced proper folding of ER tyrosinase glycoforms. Taken together, the data support a scenario in which the generation of exit-competent early glycoforms is dependent on autocatalytic activation of tyrosinase within the ER. Because autocatalytic activation is a slow process (8–10), these observations may explain the relative long delay in tyrosinase release from the ER as compared with the homologous melanogenic glycoprotein TRP1 (3, 33). Thus, tyrosinase may exert a self-regulatory mechanism ensuring continuous trafficking of properly folded protein only under conditions favorable for activity and melanin production.

If this is indeed the mechanism, then albino tyrosinase mutations that produce inactive protein should be retained in the ER, and copper binding to tyrosinase must first occur in the ER. Thus far, three loss of function TYR albino mutants have been found to accumulate in the ER (4, 5), and a copper transporter has been localized to the ER membrane (34). Furthermore, it is possible that copper loading is required across the vesicular network, as the Menkes copper transporter that has been localized to the *trans*-Golgi (35) is required for tyrosinase enzymatic activity in fibroblasts (36). Further studies will be needed to characterize the cellular localization of other albino TYR mutations and to identify the proteins responsible for copper loading and their distribution within the secretory pathway.

This activation hypothesis suggests that inactivation of tyrosinase enzymatic activity by tumor-induced changes in the microenvironment is the likely cause for ER retention. It is possible that the culprit is altered metabolic processes, such as high rates of glucose uptake and glycolysis commonly displayed in tumors (37). Cancer cells are able to overproduce lactic acid aerobically probably because of activation of lactate dehydrogenase-A (38). Accumulation of such metabolic acids can cause DOPA protonation and tyrosinase inactivation (9). The appearance of amelanotic clones in advanced primary and metastatic pigmented tumors suggests that down-regulation of tyrosinase is a frequent consequence of these metabolic changes *in vivo*, which is also reflected in the cultured cells.

Our data shed light on several observations published in the past two decades using melanoma cells from other species. Down-regulation of tyrosinase activity and conversion from highly melanotic to amelanotic cells was observed in Cloudman S91 melanoma cells grown in phenylalanine-supplemented, tyrosine-free medium (14, 39). Likewise, the supply of extracellular tyrosine and DOPA was critical for tyrosinase in Bomirski Ab amelanotic hamster melanoma cells. Tyrosine and DOPA increased the intracellular levels of tyrosinase activity and immunoreactive protein (40) and may have also affected the synthesis of additional components in melanogenesis (41). The combined experience with Bomirski Ab amelanotic hamster melanoma cells enticed Slominski and Paus (42) to suggest that tyrosine and DOPA act as hormone-like bioregulators in mammals.

The level of tyrosinase protein within melanoma cells is consequential not only for melanin production but also for melanoma cell immunity. Tyrosinase peptides are recognized by class I-restricted, melanoma-specific tumor-infiltrating lymphocytes (see for example Refs. 6 and 43). Large resources are dedicated to boosting the immune reaction of patients to this tumor with tumor-infiltrating lymphocyte vaccines (44). Continuous presentation of the relevant epitope integrated into the vaccine is critical for a robust immune response. As production of tyrosinase peptides is dependent on the level of tyrosinase

² Unpublished observations.

protein and its proteasomal degradation products, it is conceivable that dopaminergic agents can be used as a means to synchronize high production of tyrosinase peptides to optimize the response of melanoma patients to tumor-infiltrating lymphocytes or dendritic cell vaccines.

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