Regulation of Tyrosinase in Human Melanocytes Grown in Culture

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ABSTRACT Tyrosinase, the enzyme that controls the synthesis of melanin, is a unique product of melanocytes. Normal and malignant human melanocytes grown in culture were used to study the factors that regulate the expression of tyrosinase. Immunoprecipitation experiments showed that newly synthesized tyrosinase appeared as a protein with an apparent molecular weight of 70,000 that was processed to a protein with an apparent molecular weight of 80,000. Neither tunicamycin nor 2-deoxy-D-glucose inhibited this conversion, suggesting that O-glycosylation is the major biochemical event in the posttranslational modification of tyrosinase. Agents that stimulated the proliferation of normal melanocytes also stimulated tyrosinase activity. Melanocytes with low levels of tyrosinase activity synthesized less tyrosinase, processed the enzyme more slowly, and degraded it more rapidly than melanocytes with high levels of tyrosinase activity. We conclude that tyrosinase activity in cultures of human melanocytes derived from different donors is determined predominantly by its abundance.

Epidermal melanin is synthesized by melanocytes via oxidation of tyrosine. The amount of melanin in the epidermis is determined, at least in part, by the activity of tyrosinase. For example, the activity of tyrosinase is higher in foreskins taken from black babies than in foreskins taken from caucasian babies (31); and melanocytes in patients with some forms of albinism have extremely low or no detectable tyrosinase activity (17). Knowledge of factors that regulate tyrosinase is important because melanin protects the skin from harmful solar radiation. In murine melanoma cells grown in vitro the activity of tyrosinase is at least partially regulated by the intracellular levels of cyclic adenosine monophosphate (cAMP). Exposure of these cells to substances that increase the intracellular levels of cAMP also causes an increase in tyrosinase activity and melanin content (1, 6–9, 22, 33, 38). Recently, a method for propagating normal human melanocytes in vitro (5) was developed. This achievement made it possible to study the control of tyrosinase activity in nonmalignant human melanocytes and to compare their responses with those of murine and human melanoma cells. Our experiments show that the activity of tyrosinase in cultured melanocytes is stimulated by agents that stimulate the growth of the cells and is determined by the rate of synthesis, processing, and degradation of the enzyme.

MATERIALS AND METHODS

Materials: Eagle's minimal essential medium (MEM) and Ham's F-10 medium were purchased from Flow Laboratories, Inc. (McLean, VA). Sera and other medium supplements were obtained from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY). Culture dishes were purchased from Costar (Cambridge, MA). The other chemicals were obtained from the following sources: 4-*O*-methyl-12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)[1] from Chemicals for Cancer Research, Inc. (Eden Prairie, MN), cholera toxin, dibutyryladenosine monophosphate, and other reagents used in enzyme assays and buffers from Sigma Chemical Co. (St. Louis, MO), L-[^{35}S]methionine from Amersham Corp. (Arlington Heights, IL), D-[2-^{3}H]mannose, D-[1,6-^{3}H(N)]-glucosamine, [9,10-H(N)]palmitic acid, [^{32}P]orthophosphate, sodium [S^{35}]sulfate, L-3,5[^{3}H]tyrosine, and Econofluor from New England Nuclear (Boston, MA), DE52 resin from Pharmacia Fine Chemicals (Piscataway, NJ), IgGsorb from New England Enzyme Center (Boston, MA), and polyacrylamide and protein assay kit from Bio-Rad Laboratories (Richmond, CA).

Culture of Cells: Human melanocytes were grown by a method modified from that of Eisinger and Marko (5). Foreskins from newborn babies were cleaned of fat and incubated at 4°C overnight (~18 h) in MEM without calcium and supplemented with 0.25% trypsin, 1 mM EDTA, 200 U/ml penicillin, and 100 μg/ml streptomycin. The epidermis was separated from the dermis with forceps. Melanocytes were distributed at the borders of both segments and became detached after vigorous shaking for 1 min. The dissociated

[1] *Abbreviations used in this paper:* dbcAMP, dibutyryl cAMP; DOPA, L-dihydroxyphenylalanine; IBMX, isobutylmethyl xanthine; MSH, melanotropin; TCA, trichloroacetic acid; TPA, 4-*O*-methyl-12-*O*-tetradecanoyl-phorbol-13-acetate; IBMX, isobutylmethyl xanthine.

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cells were grown in Ham's F-10 medium containing 5% newborn calf or fetal calf serum, penicillin, streptomycin, and 10 ng/ml (16 nM) TPA and 10 nM cholera toxin.

Contaminating fibroblasts and keratinocytes were removed by differential attachment to the culture dish. Cells were detached from the culture flasks with 0.1% trypsin in MEM without calcium and were subcultured in growth medium devoid of TPA for 30 min at 37°C. Most fibroblasts and keratinocytes reattached to the culture flask during this period, and the floating melanocytes were replated in medium containing TPA and cholera toxin. Confluent cultures of normal melanocytes were resuspended and seeded at a ratio of 1:3 about once every month.

Human fibroblasts obtained from foreskins were grown in Ham's F-10 medium supplemented with streptomycin, penicillin, and 10% calf serum. Human melanoma cell lines (described in reference 14), obtained from Dr. A. Houghton, Memorial Sloan-Kettering Cancer Center, NY, were grown in the same medium. Murine melanoma cells were grown as described (6, 7).

Metabolic Labeling of Cells with Radioactive Compounds: Cells at 80% confluence in 55-cm^2 culture dishes were used. For labeling with [^{35}S]methionine, they first were incubated with 10 ml of methionine-free MEM supplemented with 3% dialyzed calf serum and dimethyl sulfoxide (DMSO) to improve uptake of the radiolabel. After 4 h, the medium was replaced with 1.5 ml of fresh methionine-free medium containing 60–200 μCi/ml of L-[^{35}S]methionine (1,135 Ci/mmol). The cells were incubated for periods ranging from 0.5 to 6 h and harvested immediately or at successive intervals during a subsequent "chase" with nonradioactive methionine.

For labeling with [^{3}H]mannose and [^{3}H]glucosamine, the cells were incubated in MEM with low glucose (50 mg/L) supplemented with 1.1 g/L sodium pyruvate, 3% dialyzed calf serum, and 1% DMSO. After 2 h, the medium was replaced with fresh low glucose medium supplemented with 400 μCi/ml of D-[2-^{3}H]mannose (13 Ci/mmol) or 250 μCi/ml of D-[1,6-^{3}H(N)]glucosamine hydrochloride (32.5 Ci/mmol) for 6 h.

For labeling with [^{3}H]palmitate, the cells were incubated for 24 h in growth medium supplemented with 3% dialyzed calf serum, 1% DMSO, and 200 μCi/ml of 9,10-[^{3}H(N)]palmitic acid (15.2 Ci/mmol).

For labeling with [^{32}P]orthophosphate, cells were incubated for 18 h in MEM without inorganic phosphate supplemented with 3% dialyzed serum and 300 μCi/ml of [^{32}P]phosphoric acid.

For labeling with [^{35}S]sulfate cells were incubated with sulfate-free medium supplemented with $MgCl_2$, 3% dialyzed serum, and 1% DMSO. After 3 h, the medium was replaced with fresh sulfate-free medium supplemented with 200 μCi/ml [S^{35}]sodium sulfate for 5 h.

Preparation of Cell Extracts: The procedures described below were carried out on ice. The labeled cells were washed three times with ice-cold phosphate-buffered saline (PBS) containing methionine (2 mg/ml), or glucose (1 mg/ml), scraped into test tubes, and sedimented by centrifugation. Cells were lysed with 100 μl of PBS containing 1% Nonidet P-40 (NP-40; Sigma Chemical Co., Arlington Heights, IL) and 0.1 mM phenylmethylsulfonyl fluoride and agitated on a Vortex mixer (Scientific Industries, Inc., Bohemia, NY). The lysates were centrifuged at 16,000 *g* for 15 min. To determine the specific radioactivity in protein, 10-μl aliquots were mixed with an equal amount of PBS containing 0.2% bovine serum albumin (BSA), adsorbed onto cellulose acetate filter paper (Whatman 3 MM, Whatman Inc., Clifton, NJ) incubated overnight in cold 10% trichloroacetic acid (TCA) containing methionine or glucose, and washed with cold 10% TCA and 95% ethanol. The filters were scanned in a scintillation counter with Econofluor as the scintillation fluid. Protein concentrations were determined by the Bio-Rad protein assay with BSA as the standard.

Preparation of Tyrosinase Antisera: Tyrosinase purified from Green's hamster melanomas (30) was used to immunize rabbits. 1 ml of purified enzyme solution (100 μg/ml) was emulsified with 2 ml of complete Freund's adjuvant. Aliquots of 0.7 ml were injected into each rabbit in the four foot pads and in three subcutaneous sites. These injections were repeated 6 wk later and subsequently at 3-wk intervals. Rabbits were bled monthly after the third injection. The rabbit antisera cross-reacted with human, chicken, and murine tyrosinase (Halaban, R., unpublished data). Rabbit serum obtained from Gibco Laboratories was used as a control. Immunoglobulins from control and immune serum were purified by precipitation with sodium sulfate and cation-exchange chromatography on DE52 resin.

Immunoprecipitation: The following steps were carried out on ice. Aliquots of cell lysates containing 150 μg of protein were incubated with control immunoglobulins for 30 min, and nonspecific immune complexes were removed by allowing them to bind to 40 μl of a 20% solution of formalin-treated *Staphylococcus aureus* bacteria (IgGsorb, reference 16) in PBS with 0.5% NP-40 for 30 min. The mixture then was centrifuged at 16,000 *g* for 20 min, and the bacterial pellets were discarded. In some experiments this procedure was repeated. The final supernatant fractions were divided into two portions; one portion was treated with anti-tyrosinase immunoglobulins and the other with control immunoglobulins (8 μl of each). After 2 h, IgGsorb was added, and the bound immune complexes were sedimented at 2,000 *g* for 6 min. To verify that all the enzyme was precipitated, the supernatant fractions were subjected to an additional reaction with antityrosinase immunoglobulins. In addition, aliquots of supernatant fractions were assayed for any remaining tyrosinase activity.

The pellets of IgGsorb with bound immune complexes were resuspended in PBS containing 0.5% NP-40, transferred to clean test tubes, and centrifuged. This washing procedure was repeated twice. The immune complexes were eluted from the IgGsorb by heating the pellets at 100°C for 5 min in 100 μl of sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.001% bromophenol blue. The IgGsorb was removed by centrifugation at 16,000 *g* for 10 min, and aliquots of the supernatant solution were analyzed on 7.5% polyacrylamide slab gels in 0.1% SDS (19). The gels were stained, destained, dried, and exposed to Kodak X-Omat XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C. In some experiments the gels were prepared for fluorography by incubation in Autofluor for 2 h before drying. 2-mm segments were sliced from the dried gels from relevant radioactive bands, rehydrated with 25 μl of distilled water, and digested overnight with 5 ml of 5% Protosol (National Diagnostics, Sommerville, NJ) in Econofluor at 40°C. The radioactivity in these bands was calculated by combining the radioactive counts in the peaks after substraction of background counts. Films were also scanned with an SU-8 spectrophotomer (Beckman Instruments, Inc., Electronic Instruments Div., Shiller Park, IL).

Electrophoresis on nondenaturing gels of partially purified tyrosinase from a human melanoma cell line (SK MEL 23) and immune complexes precipitated from normal melanocytes was carried out under similar conditions but without SDS. The gels were washed three times with PBS (pH 6.6) over a 30-min period and incubated with 0.2% L-dihydroxyphenylalanine (DOPA) in PBS (pH 6.8) at 37°C. After 20 min, bands corresponding to active tyrosinase became visible as the DOPA was converted to melanin.

Tyrosinase Activity and Melanization: Tyrosinase activity was measured (8, 9, 29) in cell extracts containing 2–60 μg of protein in a final volume of 200 μl in PBS containing 0.5% NP-40, 50 μM tyrosine, 50 μM DOPA, and 5 μCi/ml [3′,5′-^{3}H]tyrosine with a final specific activity of 0.5 Ci/mmol. Measurements were made in duplicate. A unit of tyrosinase was defined as the activity of enzyme that catalyzed the oxidation of 1 μmol of tyrosine in 1 min.

To measure the extent of melanization, cell extracts containing 20–150 μg of protein were heated at 90°C for 2 h in 1 ml of 0.1 N NaOH and centrifuged at 16,000 *g* for 20 min. The optical density of the supernatants was then measured at 475 nm.

Binding of β-Melanotropin to Cells: The ability of melanocytes to bind β-melanotropin (βMSH) was determined as follows. Cells were harvested with 0.5 mM EDTA in PBS, sedimented at 50 *g*, and resuspended in 5 ml of binding buffer (15 mM HEPES in serum-free Ham's F10 containing 5 mg/ml BSA, pH 6.8). The cells were again pelleted and resuspended in fresh binding buffer and then incubated for 3 h in siliconized glass vials at 15°C in a shaking water bath in the presence of either 1 nM [^{125}I]βMSH alone (to determined total binding) or 1 nM [^{125}I]βMSH and 1 μM nonradioactive βMSH (to determine specific binding). Melanotropin (MSH) was purified from pig pituitary glands by Dr. S. Lande. The [^{125}I]βMSH was prepared by the procedure of Lambert et al. (20). At the end of the incubation period, cells were centrifuged for 1 min through discontinuous (0.15 and 0.30 M) sucrose gradients in 400-μl Beckman Microfuge tubes. The supernatants were aspirated, and the tips of the tubes containing the pelleted cells were cut off and measured for radioactivity with MiniGamma counter (LKB Instruments, Inc., Rockville, MD. Human fibroblasts and murine melanoma cells were used as negative and positive controls, respectively.

RESULTS

Tyrosinase Activity and the Extent of Pigmentation in Various Cultures of Melanocytes

The extent of pigmentation in human beings differs, depending on the individual genetic background. We investigated whether such differences were also expressed in melanocytes grown in culture and whether the extent of pigmentation correlated with the level of tyrosinase activity. Tyrosinase activity and melanin content were determined in normal melanocytes cultured from the foreskins of 18 caucasian (C) and two black (B) babies and in five melanoma cell lines. The normal melanocyte cultures were maintained for at least 3

and up to 15 mo. There was a 100-fold difference in tyrosinase activity between normal melanocyte cultures having the highest (2,000 μU/mg of protein) and lowest (20 μU/mg of protein) activities, but >50% of the cultures had tyrosinase activities of between 200 and 400 μU/mg of protein. The tyrosinase activity in any particular culture correlated well with the amount of pigment extracted from the melanocytes (Fig. 1). Four of the melanoma cell lines were amelanotic and had no detectable or extremely low tyrosinase activity; one was melanotic and had an intermediate level of tyrosinase activity (see Table III).

Tyrosinase activity in the murine Cloudman S91 melanoma can be increased by exposure of the cells to agents that raise intracellular levels of cAMP, such as dibutyryl cAMP (dbcAMP), isobutylmethyl xanthine (IBMX) or MSH (1, 6–9, 22, 38). The effects of these agents on the tyrosinase activity of normal human melanocytes are shown in Table I. The agents that caused a significant increase in tyrosinase activity were TPA, IBMX, and dbcAMP. Cholera toxin had only a marginal effect and αMSH or βMSH had none. The lack of response to MSH was not due to the prolonged growth of cells in the presence of cholera toxin. Normal melanocytes grown in the absence of cholera toxin for 3 mo likewise did not respond to MSH (data not presented). The substances that stimulated tyrosinase activity also stimulated cell growth (Table I, numbers in parentheses). When TPA was not present, IBMX and dbcAMP were the only substances that maintained the short-term growth of normal melanocytes. These two substances were active only when added at the high concentration indicated in Table I; lower concentrations were ineffective (data not shown). TPA was the most effective growth stimulator, and in its presence none of the other agents caused any further increase in either the rate of growth or the activity of tyrosinase. Human melanoma cells grown in the absence of TPA and cholera toxin also did not respond to MSH. The lack of stimulation of tyrosinase by MSH was observed in normal and malignant human melanocytes expressing high or low tyrosinase activity. In contrast, tyrosinase activity in the murine melanoma cells was stimulated fivefold by the hormone.

Binding of MSH to Human Melanocytes

Although the human melanocytes did not respond to MSH, they were able to bind [^{125}I]βMSH. As shown in Table II, normal and malignant human melanocytes bound up to 30% of the radiolabeled βMSH in a specific manner. In contrast, human fibroblasts showed no specific binding. The highly responsive murine Cloudman melanoma cells specifically bound 85% of the [^{125}I]βMSH.

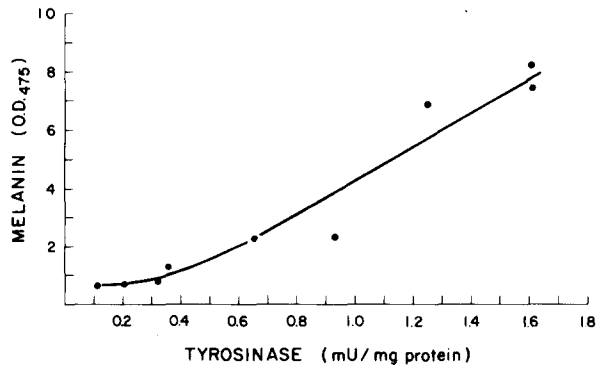

FIGURE 1 Correlation of tyrosinase activity and melanin content. Melanin is expressed as the optical density at 475 nm of a cell lysate containing 1 mg of protein/ml. Each point represents a culture of melanocytes from a different foreskin. Measurements were made in duplicate; standard error did not exceed 5%.

TABLE I

Tyrosinase Activity in Human Melanocytes Grown in the Presence of Various Substances

Additions to growth medium	Tyrosinase activity	
	No TPA	With TPA
	μU/mg protein	
None	178 (56)	473 (146)
IBMX	406 (117)	495 (149)
αMSH	184 (56)	477 (121)
CT	245 (52)	509 (119)
dbcAMP	596 (84)	403 (186)
αMSH, IBMX	454 (117)	400 (199)
CT, IBMX	526 (110)	489 (188)
dbcAMP, IBMX	627 (112)	424 (160)
CT, IBMX, MSH	651 (108)	432 (207)
CT, IBMX, dbcAMP	533 (109)	357 (212)

Normal melanocytes (C18) were grown in culture for 5 mo in the presence of TPA and cholera toxin (CT). Cells were subjected to experimental conditions for 5 d before harvesting. Numbers in parentheses give the amount of protein in micrograms at the end of the experiment. Concentrations of the various substances were as follows: CT: 10 nM; αMSH: 50 nM; IBMX: 0.1 mM; dbcAMP: 1.0 mM; TPA: 16 nM. Standard error did not exceed 10%. Values that differ by >20% are significantly different from each other.

TABLE II

Binding of [^{125}I]βMSH to Melanocytes and Fibroblasts

Cell culture	[^{125}I]βMSH bound (cpm/million cells) 1 × 10^{-3}		Specific binding	
	No MSH	With MSH	*cpm/1 × 10^{-3}*	*% of total*
Normal human melanocytes (C80)	4.9 ± 0.98	3.4 ± 0.50	1.5	30
Human melanoma cell lines				
SK MEL 13	7.8 ± 0.60	5.9 ± 1.02	1.9	24
SK MEL 23	42.8 ± 2.50	27.8 ± 0.50	15.0	35
SK MEL 28	2.5 ± 0.04	1.8 ± 0.20	0.7	28
SK MEL 37	2.5 ± 0.24	2.0 ± 0.13	0.5	20
SK MEL 127	11.9 ± 0.74	8.3 ± 0.59	3.6	30
Cloudman murine melanoma	25.3 ± 0.94	3.6 ± 0.05	21.7	86
Normal human fibroblasts	4.0 ± 0.93	4.5 ± 0.94	0	0

The assay was performed in the presence and absence of unlabeled βMSH (1 μM) as described in Materials and Methods. Values are averages of triplicates ± SD.

Immunoprecipitation of Tyrosinase with Antityrosinase Antibodies

We used antityrosinase antibodies to correlate the level of tyrosinase activity with the amount of tyrosinase protein. The binding of the antibodies raised in a rabbit against tyrosinase purified from hamster melanomas was tested with human tyrosinase. Cell extracts, prepared from normal melanocytes (C80) metabolically labeled with [^{35}S]methionine were reacted with immunoglobulins purified from either the anti-serum or control serum. The antityrosinase immunoglobulins precipitated material which on analysis by PAGE appeared as a

double band with an apparent molecular weight of 70,000–80,000. This double band was absent in control immunoprecipitates (Fig. 2 *A*). Some proteins precipitated with both the antityrosinase and the control immunoglobulins. Most of this nonspecific binding was abolished by repeated reaction of the cell extracts with control immunoglobulins (see Fig. 5). The major radioactive band of material precipitated specifically with 8 μl of a solution of antityrosinase immunoglobulins gave the highest absorbance in the scanning densitometer (Fig. 2 *B*). No tyrosinase activity was detected in any of the supernatants of cell extracts treated with antityrosinase antibodies and precipitated with IgGsorb. In contrast, all the activity remained in the supernatants after treatment with control serum (Fig. 2 *C*). Reaction of the supernatants already treated with antityrosinase antibodies with a second aliquot of antityrosinase antibodies did not result in additional precipitation of tyrosinase. In control experiments, treatment of lysates of human fibroblasts with antityrosinase antibodies did not precipitate any material corresponding to a molecular weight of 70,000–80,000 (data not shown).

The radioactive protein precipitated from normal melanocytes (C80) by the antityrosinase antibodies and a sample of tyrosinase partially purified from a human melanoma cell line (SK MEL 23) by concanavalin A-Agarose (Bethesda Research Laboratories, Inc., Gaithersburg, MD) affinity chromatography (26) were subjected to polyacrylamide gel electrophoresis under nondenaturing conditions. Fig. 3 shows that the melanoma tyrosinase visualized by reacting with DOPA and the radioactive band from the normal melanocytes have the same migration pattern.

Cellular Processing of Newly Synthesized Tyrosinase

The material precipitated by the antibodies against tyrosinase appeared to consist of two proteins (Fig. 2A). To determine whether the doublet band on the gel represented proteins having a precursor-product relationship, normal melanocytes (C80) were labeled for 30 min with [^{35}S]methionine. As shown in Fig 4, one culture was harvested immediately and the other was "chased" for 4 h with nonradioactive methionine. The results indicate that the protein with the lower molecular weight is synthesized first and is converted to a protein of higher molecular weight.

Tyrosinase is present in mammalian melanocytes in at least two, and possibly three, isozymic forms (2, 3, 11, 12, 27, 30, 35). Tyrosinase is a glycoprotein and it has been suggested that the differences in the molecular weights of the isozymes are due to differences in the content of carbohydrates (11, 12, 26). We therefore performed several experiments to determine whether the conversion of tyrosinase from the light to the heavy form includes glycosylation.

Cells were treated with two potent inhibitors of glycosylation: tunicamycin (23, 34) and 2-deoxy-D-glucose (25). The cells were exposed to tunicamycin (0.2 or 1.0 μg/ml) in the culture medium 1 h before the labeling period, during the labeling with [^{35}S]methionine, and during the chase period. Fig. 5 shows that tunicamycin did not inhibit the synthesis or processing of tyrosinase. Tunicamycin may partially inhibit the degradation of proteins, because the specific radioactivity of [^{35}S]methionine in protein was 50% higher at the end of the 3-h chase period in cells treated with the drug (0.2 μg/ml) than in controls.

2-Deoxy-D-glucose (3 mg/ml) added to the medium 3 h before and during the labeling did inhibit the synthesis of tyrosinase (Fig. 5). The specific radioactivity of [^{35}S]methionine in protein was similar in cells treated with 2-deoxy-D-

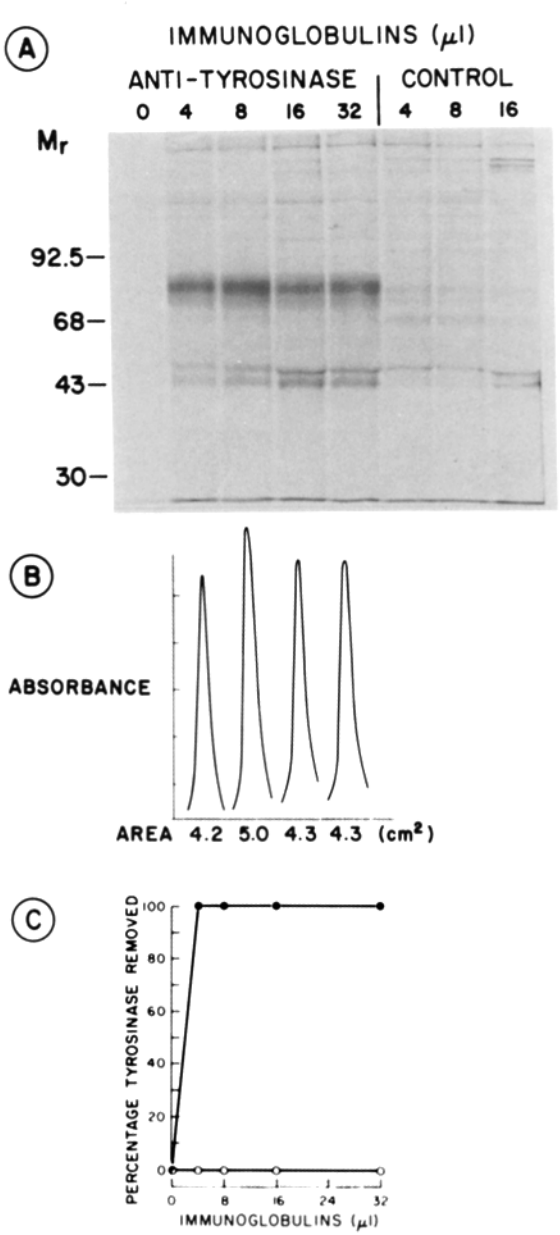

FIGURE 2 Immunoprecipitation of tyrosinase with antityrosinase antibodies. Melanocytes (C80) were incubated with medium containing 60 μCi/ml of [^{35}S]methionine for 4 h, and immunoprecipitation was carried out as described in Materials and Methods. Tyrosinase activity of the melanocytes was 950 μU/mg of protein. (*A*) Autoradiograph of 7.5% SDS polyacrylamide slab gel. The cell lysate (total of 320 μg of protein) was divided into eight equal aliquots. To one aliquot no immunoglobulins were added (0). The others were immunoprecipitated with increasing concentrations of antityrosinase immunoglobulins (4, 8, 16, and 32 μl) or control immunoglobulins (4, 8, and 16 μl). The entire immunoprecipitate was analyzed on 7.5% SDS polyacrylamide gel, which was autoradiographed for 10 d. The positions of molecular weight markers ($M_r \times 10^{-3}$) are shown on the left. They included phosphorylase b (92.5), bovine serum albumin (68), ovalbumin (43), and carbonic anhydrase (30) (Bio-Rad Laboratories). (*B*) Densitometer tracings of the radioactive bands in *A* that correspond to tyrosinase. (C) Tyrosinase activity in the supernatants after precipitation of proteins with antibodies and IgGsorb. Cell extracts were treated with antityrosinase antibodies (●) or with control immunoglobulins (○).

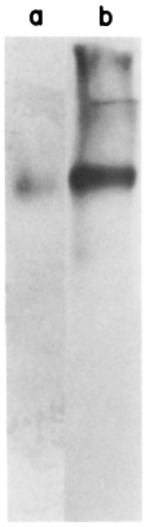

FIGURE 3 Comparison of the electrophoretic mobility of tyrosinase activity and radioactive immunoprecipitated tyrosinase. Partially purified tyrosinase from human melanomas SK MEL 23 (*a*) and tyrosinase immunoprecipitated from a lysate of metabolically labeled C80 cells (*b*) were subjected to PAGE (7.5%) without SDS. The slab gel was incubated with 0.2% DOPA, dried, and exposed to film. (*a*) DOPA reaction on gel. (*b*) Radioactive band on film.

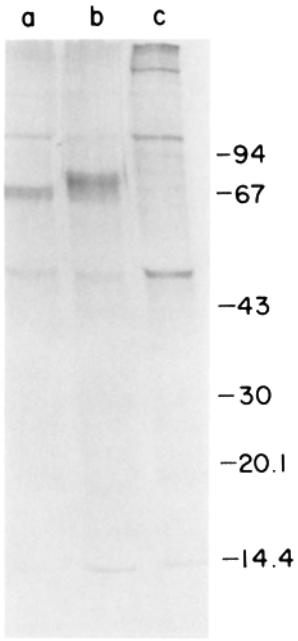

FIGURE 4 Processing of tyrosinase. Two cultures of C80 melanocytes were metabolically labeled with [^{35}S]methionine for 30 min. One culture was harvested immediately (*a*) and the other was further incubated for 4 h in growth medium containing unlabeled methionine (*b*). Cell lysates were immunoprecipitated with antityrosinase antibodies (*a* and *b*) or with control immunoglobulins (*c*). Precipitates of control immunoglobulins with lysates of pulsed or pulse-chased cells gave similar patterns of film. Immune complexes were separated on 7.5% SDS polyacrylamide slab gel, dried, and autoradiographed for 3 d. Markers ($M_r \times 10^{-3}$) included phosphorylase b (94), albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), and α-lactalbumin (14.4) (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ).

glucose and in control cells, indicating that the inhibition of synthesis of tyrosinase was not the result of a general effect on synthesis of proteins. Normal melanocytes grown for 9 d in the presence of either 2 or 4 mM 2-deoxy-D-glucose (0.33 and 0.67 mg/ml, respectively) had tyrosinase activity 35 and 22% of control, respectively. There was a slight reduction (20%) in total protein when cells were grown in the presence of 4 mM 2-deoxy-D-glucose. Therefore, the absence of precipitable radioactive tyrosinase after incubation of cells with 2-deoxy-D-glucose was the result of reduction in the amount of enzyme rather than reduction in the binding activity of the antibodies to a carbohydrate-free tyrosinase. When 2-deoxy-D-glucose was present only during the chase period, the maturation of tyrosinase into a heavier isozyme was not inhibited, but there was a small reduction in the molecular weight of the processed enzyme (Fig. 5).

To better understand the effects of tunicamycin and 2-deoxy-D-glucose on the processing of tyrosinase, we metabol-

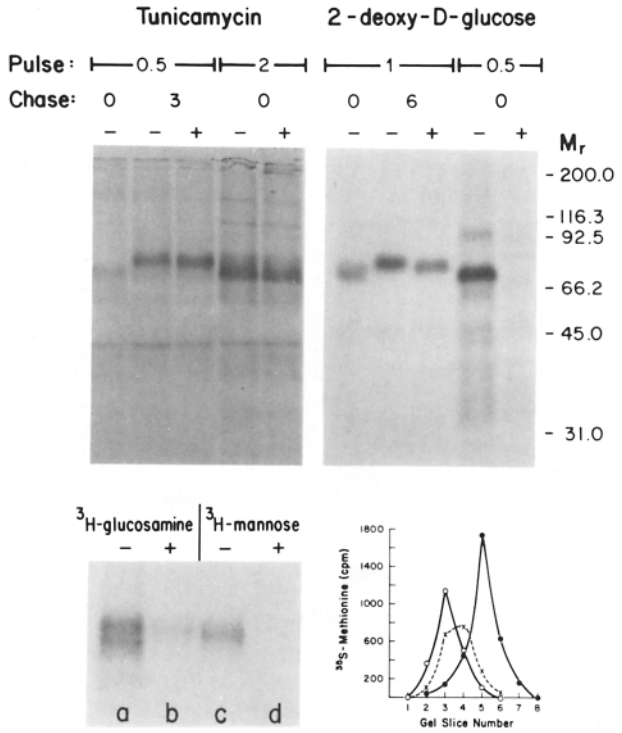

FIGURE 5 Effects of tunicamycin and 2-deoxy-D-glucose on the synthesis and processing of tyrosinase. *Top:* Normal melanocytes were metabolically labeled with [^{35}S]methionine for 0.5, 1, or 2 h (pulse). Tunicamycin, 0.2 μg/ml (+), was added 1 h before the beginning of the pulse; 2-deoxy-D-glucose, 3 μg/ml (+), was added at the beginning of the chase or 3 h before the beginning of the 0.5-h pulse. Cells were harvested either at the end of the pulse (0) or were further incubated in regular medium without the radioactive amino acid for 3 or 6 h (chase). C80 melanocyte culture was used in the tunicamycin experiments: the dried gel was autoradiographed for 3 d. C8 melanocyte cultures were used in the 2-deoxy-D-glucose experiment. The dried gel was fluorographed for 2 d. Tyrosinase activity and abundance in these melanocyte cultures are given in Table 3. *Bottom right:* Radioactivity in gel slices taken from the dried gel of the 2-deoxy-D-glucose experiment. 30-min pulse (●); 6-h chase (○); 6-h chase in the presence of 2-deoxy-D-glucose (×). The direction of electrophoresis is from left to right. *Bottom left:* Normal melanocytes (B90) were metabolically labeled with [^{3}H]glucosamine (130 μCi/ml) or [^{3}H]mannose (100 μCi/ml) for 6 h in low glucose medium in the absence or presence of tunicamycin (1 μg/ml). Cell extracts were immunoprecipitated with antityrosinase antibodies and subjected to polyacrylamide gel electrophoresis as described. The dried gel was fluorographed for 14 d. Total radioactivity in TCA-precipitable material used in each assay was 675,000 and 150,000 cpm for glucosamine and mannose, respectively. Radioactivity in the tyrosinase band was 680, 420, and 1,310 cpm in lanes a, b, and c, respectively. There was no detectable radioactivity above background in lane d.

ically labeled cells with [^{3}H]mannose or [^{3}H]glucosamine in the presence or absence of either of these two inhibitors. Tunicamycin at 0.2 and 1.0 μg/ml concentrations inhibited the incorporation of [^{3}H]mannose into TCA-preciptable material by 80 and 98%, respectively. However, it did not inhibit the incorporation of [^{3}H]glucosamine into TCA-preciptable material. In contrast, 2-deoxy-D-glucose inhibited the incorporation of both [^{3}H]mannose and [^{3}H]glucosamine by 70 and 94%, respectively. In the absence of any inhibitors, tyrosinase bound ~0.1 and 0.8% of the total radioactive glucosamine and mannose incorporated into glycoproteins, respectively. Tunicamycin abolished the incorporation of [^{3}H]mannose into tyrosinase, whereas incorporation of [^{3}H]glucosamine was reduced by only 40% (Fig. 5, bottom). These results indicate that the glycosylation of tyrosinase proceeds by two different mechanisms, one sensitive and the other insensitive to tunicamycin.

There was no incorporation of [^{3}H]palmitate, [^{32}P]phosphate, or [^{35}S]sulfate into tyrosinase (data not shown); the dried gels were exposed to the film for 95, 35, and 14 d, respectively.

Abundance, Processing, and Degradation of Tyrosinase in Melanocytes Expressing Low and High Tyrosinase Activity

To determine whether tyrosinase activity correlates with the level of tyrosinase protein, we investigated the abundance and the rate of degradation of tyrosinase in cultures of melanocytes derived from different foreskins. We chose cultures of melanocytes having low (C9 and C33), medium (C22), and high (C8, B1, and C80) activities (Table III). Cells were labeled metabolically by incubation with [^{35}S]methionine for 4 or 6 h and then harvested immediately or at various later times. The rates of incorporation of [^{35}S]methionine into protein of each culture were similar, as were the rates of degradation (data not shown).

The labeled cells were lysed and immunoprecipitations were carried out. The tyrosinase precipitated from each lysate was analysed by SDS polyacrylamide slab gel electrophoresis. These analyses are shown in Figs. 6–9. Several differences are seen between the results obtained from melanocytes having low tyrosinase activity and those obtained from melanocytes with high activity.

The cells in cultures C9 and C33, in which tyrosinase activity is low, appear to have synthesized 24–33% of the amount of enzyme produced by the cultures C8, B1, and C80, in which the level of tyrosinase activity is high (Figs. 6–9 and Table III). C33 and C9 cells also processed tyrosinase more slowly and degraded it more quickly (Figs. 6–9 and Table III). After the initial 4-h period of labeling with [^{35}S]methionine, most of the tyrosinase from C9 and C33 cells was detected only in the faster moving band of the doublet, whereas the tyrosinase from C80 cells was detected predominantly in the more slowly moving band (Figs. 6 and 9). In the experiments described above we showed that C80 cells complete the conversion of tyrosinase to the heavier isozyme within 3 h (Fig. 5), whereas C33 cells require 10 h (Figs. 6 and 7). Even at the end of a 4-h chase following a 4-h pulse, only a fraction of the label in C33 cells could be found in the slow band (data not shown). In addition, in C33 cells, 70% of the enzyme had been degraded at the end of a 10-h chase period, whereas none was degraded during this period in C80 cells (Fig. 8).

TABLE III

Activity and Abundance of Tyrosinase in Melanocyte Cultures

	Activity	Abundance		
		Labeling period	Chase	Percent of total protein
	μU/mg protein	*(h)*		
Normal melanocytes				
C9	41	4	0	0.051
		4	16	0.008
C33	69	0.5	0	0.060
		4	0	0.062
		4	10	0.017
		4	16	0.015
C22	485	0.5	0	0.170
		0.5	6	0.180
		4	0	0.108
		4	16	0.047
C80	945	0.5	0	0.200
		0.5	2	0.410
		4	0	0.204
		4	10	0.205
		4	16	0.140
B1	1,191	4	0	0.190
		4	16	0.137
C8	1,290	0.5	0	0.220
		0.5	3	0.210
Melanoma cells				
SK MEL 23	220	6	0	0.110
		6	16	0.055
SK MEL 13	16	6	0	ND
SK MEL 28	2	4	0	ND
SK MEL 37	ND	4	0	ND
SK MEL 127	62.5	6	0	0.013

Cells were metabolically labeled with [^{35}S]methionine for the time indicated and were either harvested immediately (0 chase) or incubated (chased) in regular medium for various times. Abundance of tyrosinase is given as the percent of radioactive counts in the tyrosinase band in slices of polyacrylamide gel compared with the total radioactivity of the protein used in the immunoprecipitation assay. C and B represent caucasian and black origins of melanocytes, respectively. *ND*, not detectable.

We calculated the half-life of tyrosinase from the linear phase of the slopes in Fig. 8 to be 3 h in C33 and 14 h in C80 cells; these half-life values probably reflect the rate of degradation of the low vs. high molecular weight forms of the enzyme, respectively.

The differences in the patterns of synthesis, processing, and degradation of tyrosinase were not limited to normal melanocytes. Such differences were also observed in cultures of human melanoma cells (Fig. 9 and Table III). Newly synthesized tyrosinase could not be detected in melanoma cells that had little or no measurable tyrosinase activity (Fig. 9, lanes i, j, m, and n, and Table III).

Tyrosinase in cells with low activity contained the same amount of carbohydrates as cells with high activity. Lysates made of C33 and C8 cells that had been metabolically labeled with either [^{3}H]mannose or [^{3}H]glucosamine for 6 h had the same amount of radioactive sugar incorporated into tyrosinase (Fig. 10). Scanning densitometer analysis of the radioactive tyrosinase bands indicated that the relative intensities of the bands were 1.00, 1.05, 0.28, and 1.2 for lanes *1*, *2*, *3*, and *4* (Fig. 10), respectively. Taking into account the specific radioactivity of each carbohydrate in protein and the 3.7-fold

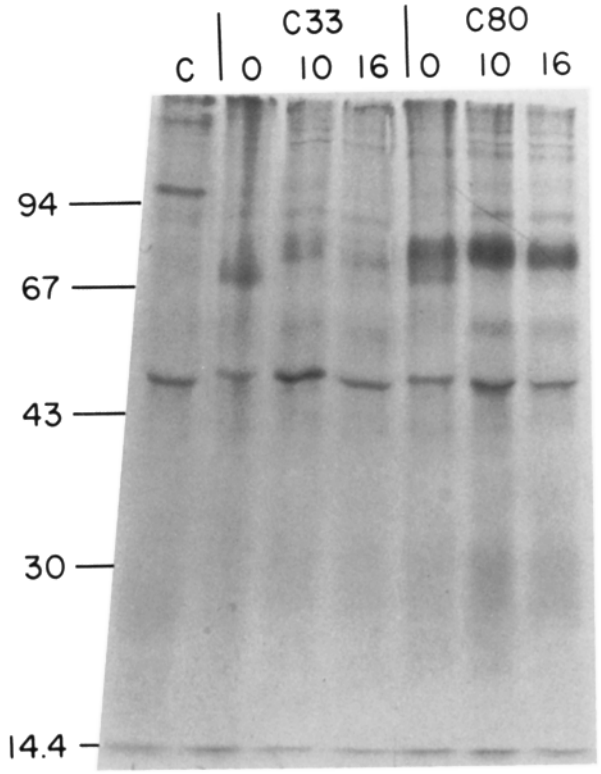

FIGURE 6 Tyrosinase in cells with low and high tyrosinase activity. Cultures of melanocytes originating from caucasian donors having low (C33) and high (C80) tyrosinase activity were labeled with [^{35}S] methionine for 4 h. They were then harvested immediately (0) or incubated in regular growth medium for 10 or 16 h. Cell lysates were immunoprecipitated with control immunoglobulins (C) or with antityrosinase antibodies (0, 10, 16). The amount of sample buffer containing the eluted protein loaded on each lane was corrected (within each cell line) to compensate for the decrease in the specific radioactivity in proteins during the chase period.

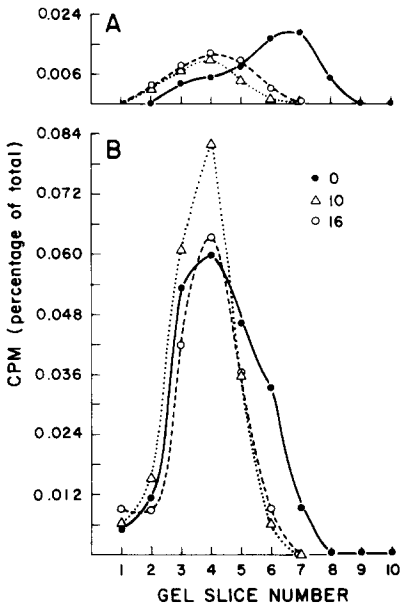

FIGURE 7 Radioactivity in immunoprecipitated tyrosinase from low and high tyrosinase cells. The tyrosinase bands from the dried gel shown in Fig. 6 were excised, rehydrated, and extracted with Protosol in Econofluor (5% vol/vol) at 40°C for 24 h to determine radioactivity. Radioactivity in tyrosinase from C33 cells (low tyrosinase activity) is shown in *A* and from C80 cells (high tyrosinase activity) in *B*. Radioactivity in each gel slice is presented as a percent of the total radioactivity in proteins used in the immunoprecipitation assay, taking into account the fraction of the sample applied to the gel. The total radioactivity in protein in the immunoprecipitation reactions ranged from 2.2 to 4.9 × 10^6 cpm. 4-h labeling period without chase (●), with 10-h (△) and 16-h (○) chase. The direction of electrophoresis is from left to right.

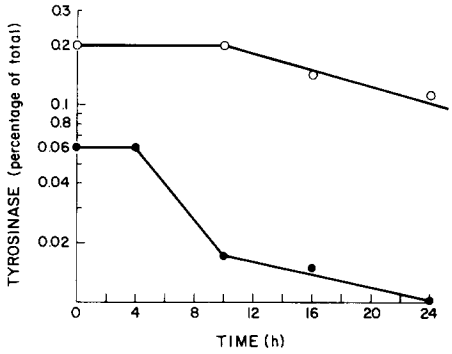

FIGURE 8 Kinetics of degradation of tyrosinase. radioactivity in tyrosinase precipitated from lysates of [^{35}S]methionine-labeled cells was calculated from the total radioactivity in all the gel slices taken from tyrosinase bands such as those shown in Figs. 6 and 7. The data are expressed as the percent of the total radioactivity in proteins used in the immunoprecipitation, taking into account the fraction of the sample analyzed on the gel. Averages from two to three experiments are presented. Standard error did not exceed 10%. High tyrosinase C80 melanocyte culture (○); low tyrosinase C33 melanocyte culture (●).

difference in the abundance of tyrosinase in C8 compared with C33 cells (Table III), it is evident that tyrosinase in C33 cells contains carbohydrate similar in amount to that in C8 cells.

DISCUSSION

Our studies on human tyrosinase give, for the first time, values for the abundance of the enzyme protein in melanocytes. Previous studies on tyrosinase used the DOPA reaction for detection of the enzyme, a technique that relies exclusively on the levels of enzyme activity.

We have shown that pigmentation of normal human melanocytes derived from different donors is expressed over a wide range, probably reflecting differences among individuals. The levels of melanization correlated positively with the levels of tyrosinase activity. The activity of tyrosinase in normal and malignant human melanocytes was determined by the abundance of the enzymes, which in turn depended on the rates of synthesis and degradation. For example, in C8 cells with 32 times more enzymatic activity, the synthesis of tyrosinase was five times greater and degradation of the enzyme was four times slower than in C9 cells. This mechanism of regulation of tyrosinase activity is not limited to human melanocytes; the rate of synthesis of tyrosinase is also increased in murine melanoma cells stimulated by MSH and dbcAMP (Halaban, R., unpublished results). In our hands, the majority of the human melanoma cell lines were amelanotic and had extremely low or no detectable tyrosinase activity, and we have not yet found a normal human melanocyte culture that did not have melanin and a detectable tyrosinase

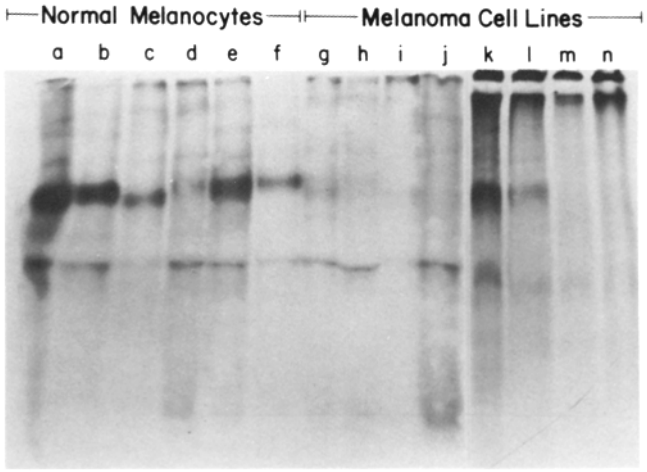

FIGURE 9 Tyrosinase was immunoprecipitated from lysates of [^{35}S] methionine-labeled cells, analyzed on a 7.5% SDS polyacrylamide gel, and fluorographed. Normal melanocytes from different foreskins and melanoma cell lines used are as follows: B1 (*a* and *b*); C9 (*c* and *d*); C22 (*e* and *f*); SK MEL 127 (*g* and *h*) SK MEL 28 (i, j); SK MEL 23 (*k*, *l*); SK MEL 13 (*m*), and SK MEL 37 (*n*). Cells were metabolically labeled with [^{35}S]methionine for 4 h and harvested immediately (*a*, *c*, *e*, *g*, *i*, *k*, *m*, and *n*) or chased for 16 h (*b*, *d*, *f*, *h*, *j*, and *l*). Tyrosinase activity of the various cell cultures is given in Table III. Dried gel was fluorographed for 1 d.

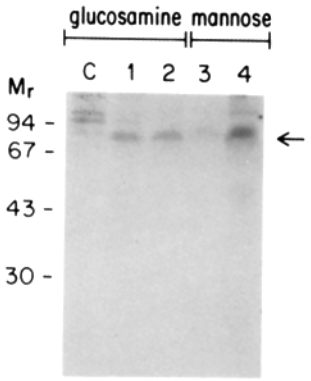

FIGURE 10 Incorporation of [^{3}H]glucosamine and [^{3}H]mannose into tyrosinase. Extracts of cells metabolically labeled for 6 h with [^{3}H]glucosamine (250 μCi/ml) and [^{3}H]mannose (400 μCi/ml) were used in immunoprecipitation assays. Cell lysates contained 200 μg of protein. Approximately 3.5 times more [^{3}H]glucosamine was incorporated into C33 than into C8 protein (1,445,000 and 411,400 cpm in protein of lanes *1* and *2*, respectively) and approximately equal amounts of [^{3}H]mannose (493,000 and 460,000 in protein of lanes *3* and *4*, respectively). The arrow indicates the position of [^{35}S]methionine-labeled tyrosinase precipitated from cells that were pulsed for 30 min with the radioactive amino acid.

activity. This finding may indicate that, as a result of transformation, some cells synthesize less tyrosinase or that amelanotic melanocytes undergo malignant transformation more frequently than their melanotic counterparts.

The newly synthesized tyrosinase of human melanocytes has an apparent molecular weight of 70,000 and is converted within hours to a species with a molecular weight of 78,000–80,000. Such conversion has also been observed in the appearance of DOPA-reactive tyrosinase in regenerating murine hair bulbs (2). The molecular weight values obtained by us for the human tyrosinase are close to, but not exactly the same as, those obtained for murine tyrosinase. The murine isozymes T_1 and T_2 have molecular weights of 81,200 and 67,200, respectively (2, 3, 12). A third form of tyrosinase, T_3 (56,900 mol. wt.), found in murine cells (12) was not detected in human melanocytes. Previous experiments (12) have suggested that the processing of tyrosinase involves glycosylation. Treatment of murine T_1 isozyme with neuraminidase reduced its molecular weight by approximately 8,000. Digestion of the neuraminidase-treated T_1 with β-galactosidase or α-mannosidase further reduces its molecular weight until it approaches that of the T_3 isozyme (11). The removal of carbohydrate residues from tyrosinase does not affect the enzymatic activity (11, 26). Furthermore, the different isozymes are antigenically similar (28) and contain similar amino acids (12). Thus, the protein components of the isozymes are similar and suffice for the full expression of enzymatic activity.

We corroborated these findings by demonstrating the incorporation of [^{3}H]mannose and [^{3}H]glucosamine into tyrosinase and provided further insight into the nature of the linkage of the oligosaccharides in tyrosinase. It has been well established that the attachment of oligosaccharide chains to peptides can be by an *N*-glycosidic linkage from *N*-acetylglucosamine to the amide nitrogen of asparagine (N-glycosylation) or by an *O*-glycosidic linkage to the hydroxyl group of serine and/or threonine (O-glycosylation) (18). Tunicamycin and 2-deoxy-D-glucose are known to inhibit N-glycosylation by blocking the attachment of the first sugar residue, *N*-acetylglucosamine to the lipid-carrier dolichol monophosphate, so that glycoproteins synthesized are deficient in asparagine-linked oligosaccharides (4, 13). Although the two inhibitors act in a similar fashion on other glycoproteins in other cellular systems, their effects on the synthesis or processing of tyrosinase were different.

It is evident from our results that oligosaccharides are attached to tyrosinase by both N- and O-linkage, because tunicamycin inhibited the incorporation of mannose but only reduced the incorporation of glucosamine into tyrosinase. Because tunicamycin did not inhibit the processing of tyrosinase to the high molecular weight species, it is evident that this conversion proceeds mostly by O-glycosylation.

The results of our experiments also indicate that there is a substantial lag time for the appearance of the high molecular weight species of tyrosinase. Thus, the processing of tyrosinase may be similar to that suggested for human chorionic gonadotropin, in which N-glycosylation occurs cotranslationally and the addition of O-linked carbohydrates to the polypeptide occurs posttranslationally in the Golgi complex (10). It is possible that the O-glycosylation of tyrosinase confers protection against degradative enzymes, because the isozyme with low molecular weight in cells with low tyrosinase activity is degraded at a faster rate than the processed enzyme in cells with high tyrosinase activity.

2-Deoxy-D-glucose inhibited the synthesis of tyrosinase without affecting the synthesis of total protein. Inasmuch as 2-deoxy-D-glucose is an analogue of glucose, it might affect the synthesis of tyrosinase by interfering with normal sugar metabolism and not through its effect on glycosylation. The synthesis of glycoproteins in human melanocytes depend on the carbon source in the medium; depriving the cells of a carbon source caused a marked decrease in the incorporation of radioactive sugars into protein (our unpublished data).

It also is clear that tyrosinase is not affected by three other posttranslational modifications, those due to the covalent

linkage of fatty acid, phosphate, and glycan residues. We detected no incorporation of palmitic acid, inorganic phosphate, and sulfate into tyrosinase.

Tyrosinase activity of normal human melanocytes was stimulated by substances, such as TPA, IBMX and dbcAMP, that also stimulated cellular proliferation. Similar findings have been reported for murine Cloudman melanoma cells (6, 7, 32). These murine cells require high levels of cAMP for maintaining a high rate of proliferation and high levels of tyrosinase activity. It has been reported that TPA caused a sixfold increase in the content of melanin in human melanoma cells grown in culture (15). In contrast to the findings in murine cells, we found tyrosinase activity and the rate of cellular proliferation of normal and malignant human melanocytes to be insensitive to MSH, regardless of whether the cells had low or high tyrosinase activity or originated from black or caucasian individuals. These were unexpected findings because the administration of repeated doses of adrenocorticotrophic hormone or αMSH to adult human subjects is known to cause cutaneous hyperpigmentation within 3 d (21, 24).

The differences between responses of melanocytes to MSH in vivo and in vitro may be due to the fact that melanocytes from newborns and malignant melanocytes do not display the full range of melanocytic surface proteins present in adults (14). Both normal and malignant melanocytes specifically bind 17–35% of [^{125}I]βMSH. This level of binding is significantly higher than that of nonmelanocytic cells, such as human fibroblasts, but is much lower than the 86% binding observed in murine melanoma cells that are highly responsive to MSH. It is not as yet clear whether this difference reflects the number of receptors present on cells of different lines or a difference in the optimum receptor-binding conditions for these lines. It is possible that the lack of response to MSH in normal and malignant human melanocytes is due to binding of insufficient numbers of hormone molecules. It also is possible that other processes known to be associated with the stimulation of melanization by MSH in murine melanomas, such as internalization of the hormone (36, 37), activation of adenylate cyclase (1, 22), and activation of protein kinase (9, 22, 33), are lacking in normal human melanocytes. It also is possible that MSH indirectly stimulates melanization in vivo through a second mediator rather than by a direct action on the melanocytes. Studies of adult human melanocytes, preferably grown in the absence of TPA and cholera toxin, are needed to determine similarities and differences in the responses to MSH of human and murine normal and malignant melanocytes.

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