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Genetics

Murine and human *b* locus pigmentation genes encode a glycoprotein (gp75) with catalase activity

(tyrosinase/albino locus/brown locus/glycoprotein gp75)

RUTH HALABAN AND GISELA MOELLMANN

Department of Dermatology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 3333, New Haven, CT 06510

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ABSTRACT **Melanogenesis is regulated in large part by tyrosinase (monophenol monooxygenase; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1), and defective tyrosinase leads to albinism. The mechanisms for other pigmentation determinants (e.g., those operative in tyrosinase-positive albinism and in murine coat-color mutants) are not yet known. One murine pigmentation gene, the brown (*b*) locus, when mutated leads to a brown (*b/b*) or hypopigmented (B^{lt}/B^{lt}) coat versus the wild-type black (*B/B*). We show that the *b* locus codes for a glycoprotein with the activity of a catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) (catalase *B*). Only the *c* locus protein is a tyrosinase. Because peroxides may be by-products of melanogenic activity and hydrogen peroxide in particular is known to destroy melanin precursors and melanin, we conclude that pigmentation is controlled not only by tyrosinase but also by a hydroperoxidase. Our studies indicate that catalase *B* is identical with gp75, a known human melanosomal glycoprotein; that the *b* mutation is in a heme-associated domain; and that the B^{lt} mutation renders the protein susceptible to rapid proteolytic degradation.**

Mutations at the structural gene for tyrosinase (monophenol monooxygenase; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) are known to reduce coat color in mice (1) and to be the cause of tyrosinase-negative albinism (2). For example, one of the murine *c* locus mutants, himalayan (c^h/c^h), produces an underglycosylated, highly labile, temperature-sensitive tyrosinase (3) because of a point mutation replacing histidine-402 with arginine (4). Another point mutation, murine albino (*c/c*), produces an enzyme highly sensitive to proteolytic degradation because of a point mutation replacing cysteine-85 with serine (5). However, little is known about the molecular genetics of tyrosinase-positive albinism, affecting approximately half of the human albino population (6), or the >50 genetic loci other than the *c* locus that affect pigmentation in mice (7).

In recent years, two melanocyte-specific cDNA clones were isolated, and both were ascribed to tyrosinase (5, 8–12). Based on deduced amino acid sequence, the two code for glycoproteins of similar size, with a membrane-spanning domain and conserved positions of cysteine and histidine (9). One of the clones was assigned to the *c* locus on mouse chromosome 7 (5, 9) and to human chromosome 11(q14–q21) (13), and the other was assigned to the brown (*b*) locus on mouse chromosome 4 (14). The mutant *b* allele confers brown coat color and the B^{lt} allele confers an almost white color to normally black mice (7). Transfected wild-type *c* locus cDNA induced tyrosinase activity and melanin synthesis in fibroblasts, amelanotic melanoma cells, albino melanocytes, and albino transgenic mice, whereas wild-type *b* locus cDNA did not (12, 15–18). Because failure to detect tyrosinase activity in the *B* locus cDNA transfectants might have been due to low levels of expression, the question still remained whether or not the *b* locus protein was another tyrosinase, as maintained by Hearing and co-workers (19), or a different enzyme critical for melanin biosynthesis.

In this report we show that the *b* locus protein is a catalase and is identical with a known human melanosomal protein, gp75. Only the protein encoded by the *c* locus has tyrosinase activity.

MATERIALS AND METHODS

Cell Cultures. Mice were obtained from The Jackson Laboratory: B10.BR (*C/C*; *B/B*; *P/P*), himalayan C57BL/6J (c^h/c^h; *B/B*; *P/P*), BALB/c (*c/c*; *b/b*; *P/P*), pink-eyed chinchilla FS/El (c^{ch}/c^{ch}; *b/b*; *p/p*), and 129/J (c^{ch}/c; *B/B*; *p/p*). Cultures of cutaneous melanocytes were established from 1-day-old mice and grown in melanocyte medium as described (20). Albino melan-c melanocytes, isolated from LAC-MF1 (*c/c*; *B/B*; *P/P*) mice (21), and brown locus mutant melan-b melanocytes, isolated from the "Q" strain of mice homozygous for the *b* mutation (21), were obtained from D. C. Bennett (Saint George's Hospital Medical School, London). BULT melanoma cells, homozygous for the B^{lt} allele, were grown in medium supplemented with PMA and dibutyryl cAMP as described (22). B16 and Cloudman S-91 murine melanoma cells homozygous for the *B* and *b* alleles, respectively, were grown in Ham's F-10 medium supplemented only with 10% horse serum, L-glutamine, and the antibiotics listed above. These cells were stimulated with 0.2 μM melanotropin and 50 μM isobutylmethylxanthine for 3–4 days prior to use. Human melanocytes were grown as described (23). NIH 3T3 murine fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics as above.

Antibodies and Competing Antigens. Polyclonal anti-tyrosinase antibodies were raised in rabbits against hamster tyrosinase (24). They precipitate human, murine, and avian tyrosinases (3, 24, 25). Antibodies against the *b* locus protein, monoclonal TMH-1 (26) and polyclonal anti-PEP1 (19), were obtained from V. J. Hearing (National Cancer Institute, Bethesda, MD). Anti-rat immunoglobulins (Dako, Santa Barbara, CA) were used as a second antibody to precipitate or immunoblot TMH-1. Anti-gp75 monoclonal antibody MEL-5 [TA99 of Thomson *et al.* (27)] was purchased from Cambridge Research Laboratory (Cambridge, MA).

Microperoxidase (MP-11, the heme portion of equine heart cytochrome *c* with amino acids 11 through 21 still attached, 91% pure, contaminants being MP-8 and MP-9) was purchased from Sigma, and human erythrocyte catalase and antibodies were from Calbiochem.

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Abbreviation: HRP, horseradish peroxidase.

PAGE of Immunoprecipitated Proteins and Immunoblotting. Confluent cultures of melanocytes in 8-cm^2 Petri dishes were metabolically labeled with L-[^{35}S]methionine [Amersham; 1450 Ci/mol; 300 μCi/ml (1 μCi = 37 kBq)] for 1–5 hr as described (24). In some experiments, Tran^{35}S-label (ICN; 1095 Ci/mmol) was used. The procedures for immunoprecipitation and $NaDodSO_4$/PAGE (10% or 12.5% polyacrylamide) were as described (3, 24).

Nondenaturing gels were used for staining of tyrosinase and for immunoblotting. $NaDodSO_4$ was omitted from the gels and electrophoresis buffer, and the gels (7% polyacrylamide) were prerun overnight at 30 V. Cell extracts were prepared in phosphate-buffered saline (PBS; 0.01 M phosphate/0.15 M NaCl, pH 7.4) containing 1% Nonidet P-40 (NP-40) and mixed 1:1 with 2× sample buffer (3, 24) lacking $NaDodSO_4$ and 2-mercaptoethanol. Approximately 100 μg of protein was loaded in each well and electrophoresed at 4°C for 6 hr. Staining for tyrosinase activity, visible within 10 min, was performed as described (24).

Electrophoretic transfer of proteins to nitrocellulose membranes and immunoblotting were as described (28). Antibodies were detected with ^{125}I-labeled protein A (1 μCi/ml; ICN; 35 mCi/mg).

Immunoprecipitation of Enzymatically Active Tyrosinase and Catalase. Melanocyte extracts were prepared as described (24). Aliquots of 50–300 μg of protein in a final volume of 100 μl were incubated with antibodies for 2 hr at 4°C. For immunoprecipitation of catalase, NaN_3 was removed from the antibody solutions by dialysis or by repeated dilution and ultrafiltration through a Centricon-10 microconcentrator (Amicon) and from Affi-Gel protein A (Bio-Rad) by repeated washing of the beads with PBS. Aliquots of 30–100 μl of IgGsorb solution (The Enzyme Center) or Affi-Gel protein A beads were added, and the mixtures were shaken for 30 min at 4°C and then centrifuged at 6000 × g for 2 min. The pellets were assayed directly for tyrosinase activity after two washes with 1 ml of 1% NP-40 in PBS. For catalase assays, immunocomplexes were eluted from the IgGsorb or Affi-Gel protein A with 300 μl of diluted (1:10) and acidified (with HCl) PBS (pH 3.0) or with Bio-Rad elution buffer (Affi-Prep protein A MARS II; Bio-Rad). The eluted material was immediately brought to pH 7.0 with 0.1 M Tris buffer (pH 9.0). Eluted immunocomplexes were tested directly or after having been concentrated 4-fold with a Speed-Vac.

The tyrosinase assay (tyrosine hydroxylase) was performed by the method of Pomerantz (29). Formation of dopachrome was followed in parallel tubes by measuring the A_{475} of IgGsorb immunocomplex supernatants. All assays were done in duplicate, and data are given as means, each representing one of two to eight experiments.

The assay for catalase was adapted from the competition assay of Walicke *et al.* (30). In this reaction, 3,3',5,5'-tetramethylbenzidine (TMB; dihydrochloride salt from Sigma) is oxidized to a colored product in the presence of horseradish peroxidase (HRP; Sigma) and H_2O_2. The color intensity depends on the level of H_2O_2. Preincubation of the H_2O_2 solution with commercial catalase or a catalase-containing experimental sample reduces the amount of H_2O_2 available for oxidation on addition of HRP and reduces the intensity of color. TMB was used as a filtered saturated solution in 0.25 M sodium acetate (pH 5). Fifteen microliters of cell extract, eluted immunoprecipitate, or human erythrocyte catalase (Sigma) were incubated with 8 μl of H_2O_2 (1.25 mM fresh stock solution) for 20–25 min at room temperature. HRP (50 ng per assay) was then added (50 μl), followed by the addition of 0.35 ml of TMB solution. Absorbance was recorded after 5–20 min in a Varian DMS80 spectrophotometer at 450 nm. To be able to attain a 450-nm maximum, acetate is required in the assay mixture (31).

RESULTS

Immunoprecipitation of Metabolically Radiolabeled *c* and *b* Locus-Encoded Glycoproteins. The individuality of tyrosinase and the *b* locus protein was explored by comparing immunoprecipitated radiolabeled proteins from wild-type and mutant melanocytes (Fig. 1). The data confirm published results on the nature of the mutant tyrosinase in himalayan (c^h) melanocytes (3), show the albino tyrosinase in melan-c melanocytes (c), and reveal a difference in structure between the products of the mutant *b* and wild-type *B* loci. Anti-tyrosinase antibodies precipitated underprocessed and fully processed proteins of 64 and 80 kDa from B10.BR melanocytes (wild-type *C* allele) and only a 64-kDa protein from himalayan, melan-c, BALB/c, and FS/E1 melanocytes (c^h and *c* mutant alleles). A 47-kDa tyrosinase degradation product (3) is present in the first three cell lines.

Comparative precipitations of *b* locus protein from eight different melanocyte lines, each homozygous for either the *B* or the *b* allele, showed that TMH-1 recognized two phenotypically different proteins segregating with the brown genotype (Figs. 1 and 2). A 64- to 84-kDa protein was precipitated from cells homozygous for the wild-type *B* allele, and a 77-kDa protein, appearing as a faint band, was precipitated from melanocytes homozygous for the mutant *b* allele. No protein was precipitated from NIH 3T3 fibroblasts with TMH-1, anti-PEP1, MEL-5, or anti-tyrosinase antibodies (data not shown).

Evidence That the *b* Locus Protein Is Identical with gp75. Detection by anti-tyrosinase antibodies of translation products of melanocyte-specific cDNA clones that do not code for tyrosinase (14, 32) and cross-reactivity of a monoclonal antibody raised against human melanosomal gp75 with tyrosinase (32) suggested that several melanocyte-specific proteins shared an epitope. To determine the degree of cross-reactivity between tyrosinase, the *b* locus protein, and gp75, the supernatants from the first immunoprecipitations in Fig. 1 were subjected to a second immunoprecipitation with anti-tyrosinase antibodies (Fig. 2*A*) followed by a third round with anti-gp75 antibody MEL-5 (Fig. 2*B*). The data show that TMH-1 had removed approximately half of the radiolabeled tyrosinase from the original cell extracts, but a discernible tyrosinase band was not recovered from TMH-1 precipitates (Fig. 1), not even in the case of the himalayan mutation in which a more rapidly migrating tyrosinase was separated from and not masked by *B* allele protein (Fig. 1, Himalayan lanes, compare lanes anti-tyr and TMH-1). These results suggest that in contrast to the *b* locus protein, tyrosinase was only loosely bound to TMH-1 and was easily dislodged by

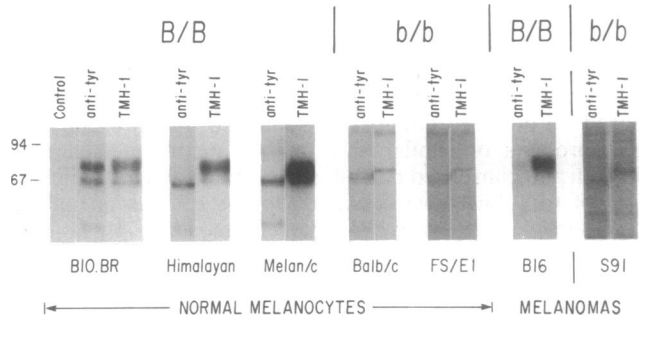

FIG. 1. Tyrosinase and *b* locus protein in wild-type and mutant melanocytes. Normal melanocytes and melanoma cells were metabolically labeled with [^{35}S]methionine for 3 and 5 hr, respectively. Immunocomplexes were resolved by $NaDodSO_4$/PAGE followed by fluorography. Control rabbit serum (control), antityrosinase antibody (anti-tyr), and TMH-1 antibody were used at dilutions 1:100, 1:200, and 1:2.5, respectively. Molecular-size markers, given in kDa, are phosphorylase *b* and albumin.

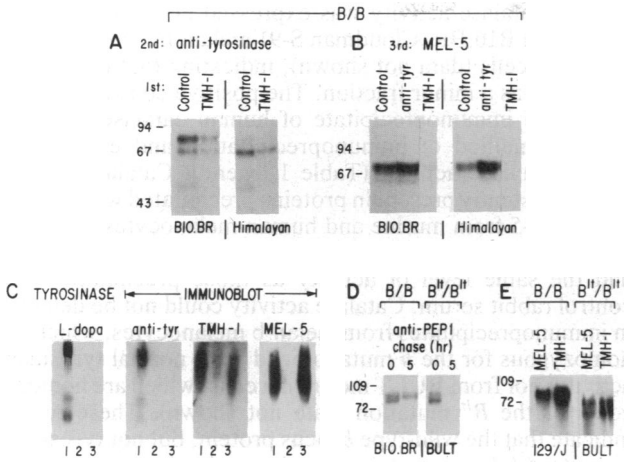

FIG. 2. Immunological cross-reactivity between *b* locus protein, tyrosinase, and gp75. (*A*) Fluorograph of tyrosinase. Supernatants of the first immunoprecipitation (Fig. 1) were subjected to a second immunoprecipitation with anti-tyrosinase antibodies. (*B*) Fluorograph of gp75. Supernatants of the second immunoprecipitation (*A*) were subjected to a third immunoprecipitation with MEL-5 (anti-gp75) at 1:100 dilution. (*C*) Tyrosinase activity and immunoblot of nondenatured proteins. Cell extracts from B10.BR (*B/B*; lanes 1), Cloudman S-91 melanoma (*b/b*; lanes 2), or melan-c (*B/B*; lanes 3) were electrophoresed in nondenaturing polyacrylamide gels. Staining for tyrosinase activity (dopa oxidase) was with L-dopa. The immunoblot is shown as an autoradiograph; anti-tyrosinase (anti-tyr), TMH-1, and MEL-5 antibodies were used at dilutions of 1:250, 1:10, and 1:100, respectively. (*D*) Brown locus protein in pulse–chase experiment. Melanocytes were metabolically radiolabeled for 1 hr. One culture was then harvested immediately (lanes 0), and the other was harvested after a 5-hr incubation in growth medium containing unlabeled amino acids (lanes 5). (*E*) Brown locus protein in 129/J and BULT melanoma cells. Cells were metabolically labeled for 4 hr. Prestained molecular-size markers, given in kDa, are phosphorylase *b* and albumin.

washing the immune complexes. Indeed, immunoprecipitation on supernatants collected from washes of IgGsorb–TMH-1 immunocomplexes confirmed that twice as much tyrosinase was released from TMH-1 as compared with control rabbit serum (data not shown).

After the third immunoprecipitation (Fig. 2*B*), it became clear that the *b* locus protein was far more similar to gp75 than to tyrosinase. In two different *B*-allelic cell lines, B10.BR and himalayan, gp75 was absent after immunoprecipitation with TMH-1 but not with anti-tyrosinase or control antibodies. Immunoblotting of natural gels containing proteins from *B/B* and *b/b* melanocytes (Fig. 2*C*) and immunoprecipitation of proteins from melanocytes homozygous for the *b* or B^{lt} alleles (Fig. 2*E*) further ruled out differences between gp75 and the *b* locus protein. Anti-tyrosinase antibodies recognized proteins with dopa oxidase activity, representing monomeric and possibly aggregated forms of tyrosinase (Fig. 2*C*, compare L-dopa lane 1 to anti-tyr lane 1), whereas TMH-1 (anti-*b* locus protein) and MEL-5 (anti-gp75) recognized proteins different from tyrosinase but mutually similar, with electrophoretic mobilities indicating approximately twice the size of the monomeric form (84 kDa) of the *B* allele protein. Binding of TMH-1 and MEL-5 to Cloudman S-91 proteins, homozygous for the *b* allele, was diminished or nonexistent, respectively (Fig. 2*C*, compare lanes 2 to lanes 1 and 3). Likewise, MEL-5 did not precipitate any protein from Cloudman S-91 cell extract (data not shown).

Melanocytes with the B^{lt} allele express a *b* locus protein highly sensitive to degradation, as shown by pulse–chase experimentation (Fig. 2*D*). After 5 hr, *b* locus protein was hardly detectable in BULT (B^{lt}/B^{lt}) melanoma cells, whereas most was still intact in wild-type B10.BR (*B/B*) melanocytes (Fig. 2*D*). TMH-1 and MEL-5 precipitated indistinguishable, probably partially degraded proteins from B^{lt}/B^{lt} melanocytes, which migrated faster than the wild-type protein (Fig. 2*E*). These results confirm that the B^{lt} mutation is indeed allelic with the *b* locus and are highly indicative that gp75 is the *b* locus protein.

The mutant *b* locus protein in Cloudman S-91 melanoma cells is deficient in a carbohydrate epitope known to be recognized by MEL-5 in human pigment cells (33). In the wild type, this epitope appeared to be added early in processing and to be removed later. Only early processed protein was precipitated by this antibody from cells radiolabeled for 25 min (data not shown), and after 3–5 hr of labeling, MEL-5-precipitated protein appeared as a narrower band of slightly lower molecular size than that precipitated by TMH-1 (compare bands in Fig. 2*E*, lanes 129/J and Fig. 3).

Partial Identification of the TMH-1 Epitope as a Heme-Associated Domain. Because the deduced amino acid sequence of the *b* locus protein suggested the presence of metal-binding sites (8) and because we had evidence that the *b* locus protein was a hydroperoxidase (see below), we tested whether heme would compete for TMH-1. Fig. 3 shows that substantial amounts of the protein recognized by TMH-1 were displaced by the presence of heme in the form of microperoxidase. The interaction appeared to occur via the TMH-1 and not the MEL-5 epitope.

Immunoprecipitation of Active Tyrosinase and Catalase. Tyrosine hydroxylase was removed from melanocyte extracts (data not shown) and recovered in immunoprecipitates (Table 1) consistently with only anti-tyrosinase antibodies. TMH-1 at high concentrations removed ≈30–40% of tyrosinase activity from supernatants in only two of eight experiments, but none was recovered in immune complexes. MEL-5 did not remove tyrosinase activity from supernatants over and above that removed by control serum, and there was no tyrosinase activity in immunoprecipitates at levels significantly different from those of controls. These data, obtained with B10.BR melanocytes, were reproduced with B16 murine melanoma cells (data not shown). Like tyrosine hydroxylase, the dopa oxidase activity of tyrosinase was present only in immune complexes formed with anti-tyrosinase antibodies (Table 1). The absence of tyrosinase activity in gp75 is in agreement with data published by others (27). Thus, it appears that only the *c* locus protein (tyrosinase), but not the *b* locus protein, catalyzes the oxidation of tyrosine to dopa and dopa to dopa quinone.

The combination of a generous histochemical peroxidase activity in melanosomes (34) and high abundance of the *b*

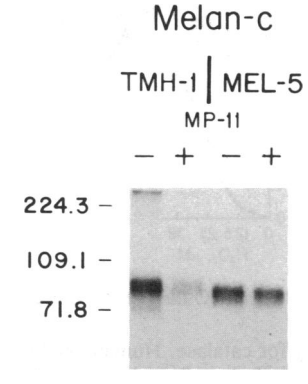

FIG. 3. Immunocompetition with microperoxidase. Melan-c melanocytes were metabolically radiolabeled for 5 hr, and extract was subjected to immunoprecipitation by TMH-1 or MEL-5 (anti-gp75) antibodies at 1:2.5 and 1:100 dilution with (lanes +) or without (lanes −) 10 μg of microperoxidase MP-11 per assay; in MP-11, the heme is attached to 11 amino acids of the heme-binding domain of cytochrome *c*. Molecular mass is shown in kDa.

Table 1. Tyrosinase and catalase activities in immunocomplexes

Antibodies	Tyrosinase in B10.BR melanocytes*		Catalase in B10.BR and human melanocytes,† A_{450}	
	Tyrosine hydroxylase, pmol/assay	Dopa oxidase, A_{475}	B10.BR	Human
Control	0.1	0	−0.011	0.007
Anti-tyrosinase	33‡	0.07§	−0.020	NT
Anti-PEP1	0.1	0	−0.275	NT
TMH-1	0.2	0	−0.634	−0.357
Anti-gp75	0.1	0	NT	−0.601

*Fifty micrograms of protein in murine B10.BR melanocyte extract was used for each immunoprecipitation. Tyrosine hydroxylase activity (formation of ^{3}HOH) in washed immunocomplexes bound to IgGsorb was measured after 105 min (or 20 min, ‡) at 37°C. Dopachrome formation was measured after 100 min (or 30 min, §) at 37°C. At the end of the reaction, IgGsorb was pelleted, and the absorbance of the supernatants was determined at 475 nm.

†Extracts of murine melanocytes (B10.BR, 300 μg of protein) and human melanocytes (Black, 230 μg of protein) were used for each immunoprecipitation. Immunocomplexes were eluted and assayed as described. Mixtures containing HRP and H_2O_2 without immunocomplexes, producing maximum color and an A_{450} of 746 and 602 for B10.BR or human melanocytes, respectively, were used as the zero reference. Immunoprecipitated human catalase, assayed alongside tubes containing eluted antigen of human melanocytes, reduced the amount of H_2O_2 to an undetectable level, abolishing color development and thus giving an A_{450} reading of −0.602. NT, not tested. Consult Fig. 4 for other details.

locus protein was an indication that the latter might be a melanosomal hydroperoxidase. Colorimetric procedures for detecting peroxidase activity in cell extracts (35–38) by which HRP was detected with ease failed to detect activity in melanocyte extracts (data not shown). In contrast, procedures for catalase readily demonstrated activity.

The colorimetric competition assay detects catalase at a wide range of concentrations (Fig. 4). At high concentration catalase diminishes and at low concentration slightly augments the color change effected by HRP, resulting in reduced and enhanced absorbance, respectively. Absorbance at 450 nm was directly proportional to the concentration of H_2O_2 when supplied between 6 to 38 μM (Fig. 4 *Inset*).

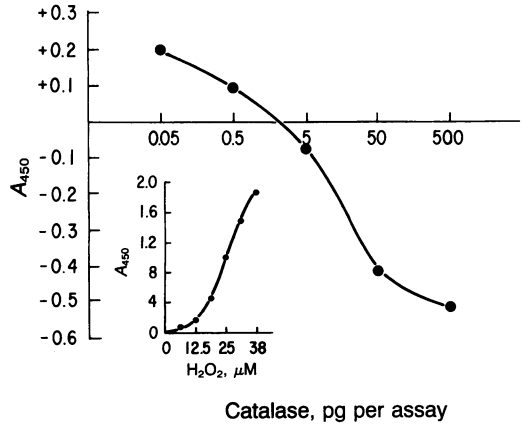

FIG. 4. An assay for catalase. Human erythrocyte catalase was incubated with 20 μM H_2O_2 in 0.4 ml of saturated TMB solution in 0.25 M sodium acetate (pH 5) for 15 min, followed by the addition of 50 ng of HRP. Absorbance was measured 5 min later; reaction mixtures without catalase (i.e., with full color development) were used as reference. (*Inset*) Absorbance at different concentrations of H_2O_2. A solution containing 50 ng of HRP and TMB without catalase and H_2O_2, (i.e., without any color development) was used as reference.

Total catalase activity was expressed at similar levels in extracts of B10.BR, Cloudman S-91 melanoma, melan-c, and NIH 3T3 cells (data not shown), indicating that the *b* locus catalase was a minor fraction. The positive control assay on the eluted immunoprecipitate of human catalase indicated that the method of immunoprecipitation and elution preserved catalase activity (Table 1 legend). Catalase activity was consistently present in proteins precipitated with TMH-1 and MEL-5 from murine and human melanocytes (Table 1). Proteins immunoprecipitated with anti-tyrosinase antibodies had the same level of activity as those precipitated with control rabbit serum. Catalase activity could not be detected in immunoprecipitates from melan-b melanocytes, which are homozygous for the *b* mutation and have normal tyrosinase activity, nor from BULT melanoma cells, which are homozygous for the B^{lt} mutation (data not shown). These results indicate that the wild-type *b* locus protein, but not tyrosinase or mutant *b* locus proteins, catalyzes the decomposition of H_2O_2 and show that gp75, like the *b* locus protein, is a catalase, confirming the identity of the two proteins as discussed earlier. We renamed the *b* locus protein/gp75 catalase *B*.

DISCUSSION

Catalase *B*. The experiments described here show the presence of a melanocytic glycoprotein with catalase activity and raise the possibility that this enzyme performs an important function in controlling melanin content through enzymatic decomposition of H_2O_2 and possibly other hydroperoxides that would disrupt melanin precursors and melanin. H_2O_2 has been shown to be produced during autooxidation of melanin precursor indoles by oxygen (39), and addition of catalase to tyrosinase reaction mixtures *in vitro* increases the yield of melanin (40). The latter experiments were done with crude preparations of mushroom tyrosinase incubated in a stream of oxygen and do not necessarily reflect on mammalian tyrosinase activity *in vivo*. However, H_2O_2 may well be liberated in melanosomes through the activity of peroxisomal oxidases as well as during the conversions of dopa to dopa quinone and 5,6-dihydroxyindole to indole-5,6-quinone catalyzed by tyrosinase (41). Absence of catalase *B* in *b* mutant melanocytes and concomitant brown instead of black coat color are indirect evidence that melanogenesis is regulated through peroxide levels in melanosomes, the subcellular organelles to which the two proteins tyrosinase and catalase *B* (gp75) have been localized by ultrastructural immunocytochemistry (33, 42).

The results show that human gp75 is a *b* locus protein and that only the protein that is recognized by the anti-tyrosinase antibodies has activities ascribed to tyrosinase. Our conclusion is in agreement with limited amino acid sequence analysis of human gp75 peptides, showing 90% identity with murine *b* locus protein (52).

The Heme or Porphyrin Domain, Homology with Tyrosinase and the *b* and the B^{lt} Mutations. TMH-1 was shown here to react with heme or a porphyrin-bound peptide because microperoxidase competed with catalase *B* for the antibody. The peptide backbone of the heme-binding site of cytochrome *c*, used here in the form of microperoxidase for immunocompetition, contains the sequence -Cys-Ala-Gln-Cys-, with the cysteines in contact with the heme side chains (43). The deduced amino acid sequences of catalase *B* and tyrosinase show two such conserved cysteines, each with two intervening amino acids, at positions 86–89 and 234–237 of the *b* locus protein (8), at positions 81–84 and 225–228 in human tyrosinase, and at 82–85 and 226–229 in murine tyrosinase (5, 9, 11, 15). (Amino acid numbering of human and murine tyrosinases of refs. 5, 10, and 12 was adjusted here to accommodate the sequence analysis of Wittbjer *et al.*; ref. 44.) The product of the mutant *b* allele appears to be altered in its heme-associated domain because it had lowered affinity for

TMH-1. Indeed, the mutation in *b/b* mice was recently identified as a replacement of Cys-86 with tyrosine (53). A conformational change conferred by this mutation is probably the reason for the deficiency in glycosylation as demonstrated by diminished molecular size and absence of MEL-5 binding. A point mutation in tyrosinase in the murine albino BALB/c, replacing Cys-85 with serine (5, 11), renders the protein susceptible to proteolysis (3). Therefore, these conserved domains must be important for catalase *B* and tyrosinase.

Catalase *B* and tyrosinase share homology with regard to 10 other positions of cysteine with flanking amino acids and with regard to seven positions of histidine (9, 45). Histidines were suggested to bind the copper in tyrosinase (46) and were used to argue that the *b* locus protein was, like tyrosinase, a copper-binding enzyme (45). However, histidines are also ligands for iron in heme. For example, an invariant sequence of -Leu-Xaa-Xaa-His- in plant peroxidases is in common with the proximal heme-binding site of globin (47). A similar segment of histidine and invariant leucine, spaced by two residues, is present in catalase *B* (amino acids 197–199) and human and murine tyrosinases (amino acids 189–192 and 191–194, respectively). We suggest, therefore, that the conserved cysteines spaced by two amino acids and the segment -Leu-Xaa-Xaa-His- act as ligands for heme and that tyrosinase and catalase *B* either bind both copper and heme similarly to bovine cardiac cytochrome *c* (48) and cytochrome *c* oxidase (49) or share homology through a potential porphyrin-binding site.

The B^{lt} mutation is a semidominant trait that causes clumping, irregular distribution, and reduced numbers of melanosomes, followed by premature death of follicular melanocytes (50). Our study shows that the *b* locus protein is inactive in B^{lt} melanocytes and that the mutation causes an increased sensitivity to proteolytic degradation. The degraded protein may release iron and thus cause an increase in free radicals known to be highly destructive to cells. Western blots of natural proteins showed that undenatured catalase *B* existed as a homodimer and also as larger aggregates. Other catalases exist in tetrameric form, important for preservation of catalatic activity (51). It is possible that in heterozygous B^{lt}/B mice, mutant catalase pairs with wild-type enzyme and forms an inactive complex.

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