High-Molecular-Weight Forms of Tyrosinase and the Tyrosinase-Related Proteins: Evidence for a Melanogenic Complex

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Tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2, (TRP-2, dopachrome tautomerase) were shown by immunoblotting and enzyme assays to copurify from extracts of Cloudman S91 melanoma cells. Antibodies to TRP-1 and TRP-2 immunoprecipitated tyrosinase activity, suggesting a stable interaction (complex) among these proteins. The tyrosine hydroxylase activity of tyrosinase was reduced in the complexed form; treatment with Triton X-100 dissociated the complex and activated the tyrosinase present within it. To further study this complex, we employed sucrose gradient density centrifugation of extracts from cultured murine melanocytes. Tyrosinase, TRP-1, and TRP-2 all existed in high molecular weight "multimers" of ~200 to >700 kilodaltons. Extraction of cells with buffers containing the detergent CHAPS preserved the high molecular weight multimers; Triton X-100 caused their dissociation into monomers. Low pH, low ionic strength, and millimolar concentrations of calcium ions favored the maintenance of multimers. The results of this study demonstrate that the participation of tyrosinase, TRP-1, and TRP-2 in a multimeric complex could have important physiologic consequences, and raise the possibility that some of the well-known interactions between coat color genes may be explained by intermolecular interactions between the gene products. *Key words: melanosome/glycoprotein/calcium/acidification. J Invest Dermatol 103:196–201, 1994*

Tyrosinase (E.C.1.14.18.1) and the tyrosinase-related proteins (TRPs) TRP-1 and TRP-2 form a family of melanosomal glycoproteins [1,2]. Each shares ~40% identity with the other two at the amino acid level, with preservation of potential copper and heme binding sites, and of cysteine residues implicated in disulfide bonding. Cloning of the cDNAs encoding these three proteins [2–4] has enabled Hearing and colleagues [5–7] to generate immunologic probes specific for each protein.

Recent studies on organisms as divergent as mammals and yeast have emphasized the importance of multimeric complexes in the biogenesis and function of subcellular organelles [8,9]. It has long been recognized that high-molecular-weight forms of tyrosinase exist [10–13]. In the past, however, relatively little attention was paid to these forms; the goal was to optimize conditions to extract monomeric tyrosinase for electrophoretic analysis and purification. While attempting to purify TRP-2 (dopachrome tautomerase; E.C.5.3.2.3) from murine melanoma cells, we noted that TRP-2, as well as TRP-1 and tyrosinase, eluted in the void volume of a high-performance liquid chromatography (HPLC)-molecular sieve column (exclusion Mr > 200 kilodaltons [kD]). We were thus prompted to characterize the high-molecular-weight forms of these three proteins and to identify the conditions that preserved their intermolecular association. Our findings, based on sucrose gradient density centrifugation of detergent extracts of cultured murine melanocytes, are described in this report.

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MATERIALS AND METHODS

Cell Lines Cloudman S91 "PS-1-HGRPT-1" cells [14] were cultured in monolayer in the presence of 100 nM β-melanocyte-stimulating hormone as described previously [15]. Cultured melanocytes from C57B16 mice (melan-a cells) were obtained from Dr. D. Bennett (London, UK) and cultured as described [16].

Purification of Dopachrome Tautomerase All purification procedures were carried out at 4°C unless otherwise noted. Cloudman S91 cells (11×10^8, 8 g wet weight) were pelleted by centrifugation, and lysed with the addition of 10 vols lysis buffer (10 mM sodium phosphate, pH 6.8; Triton X-100, 1% vol/vol; glycerol, 5% vol/vol; aprotinin, 15 μg/ml; leupeptin 15 μg/ml; and phenylmethyl sulfonyl fluoride, 1 mM) by gentle stirring (30′). The lysate was centrifuged (20,000 × *g*, 15′), the supernatant fraction (90 ml) was mixed with 30 g (wet weight) $CaPO_4$, and the mixture was stirred for 5–15 h. The mixture was then centrifuged (1000 × *g*, 10′), the supernatant fraction was dialyzed against lysis buffer and then applied to a diethylaminoethyl (DEAE) Cellulose column (BioRad, 2 × 40 cm) equilibrated with sodium phosphate (10 mM, pH 6.8) and glycerol (5% vol/vol). A linear gradient of 400 ml sodium chloride (0–400 mM) in column buffer was then run through the column and fractions (6–7 ml) were collected. Fractions containing dopachrome tautomerase and tyrosinase were located by assaying aliquots for the activities of these enzymes. Fractions from a single peak, containing both enzyme activities, were pooled, dialyzed, and concentrated by application to a small DEAE column (3 ml) and elution with column buffer containing 0.4 M NaCl (1 ml). The concentrated eluate was then applied to two Waters Protein Pak 125 high-performance liquid chromatography (HPLC) columns connected in series, equilibrated with sodium phosphate (5 mM, pH 6.8) and glycerol (5% vol/vol). Fractions (1

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Table I. Copurification of Tyrosinase and Dopachrome Tautomerase[a]

Purification Step	Tyrosinase		Dopachrome Tautomerase		Total Protein (mg)
	Total Activity (%)	Specific Activity (cpm/mg protein)	Total Activity (%)	Specific Activity (units/mg protein)	
Cell lysate	100	4.9×10^4	100	2.1	590
$CaPO_4$	122	4.5×10^6	97	154	7.8
DEAE	44	18.6×10^6	38	573	0.8
HPLC					
Peak I	6	9.9×10^6	34	1981	0.2
Peak II	21	$> 7.1 \times 10^7$	0	0	< 0.1

[a] Tyrosinase and dopachrome tautomerase were purified from 11×10^8 Cloudman S91 melanoma cells (8 g wet weight) as described in *Materials and Methods*. The final purification step (HPLC gel filtration, Fig 1) resulted in two tyrosinase peaks. Protein content of the second peak was below the level of detection of the assay.

ml/min) were collected and assayed for tyrosinase and dopachrome tautomerase activities, respectively.

Under these conditions, the dopachrome tautomerase activity of TRP-2 migrated in the void volume (Mr ≥ 200 kD) along with 20% of total tyrosinase activity [17,18]. In contrast, 80% of tyrosinase activity migrated at a position in the column corresponding to its monomer (~75 kD) molecular weight [17,18].

Density Centrifugation Cultured melan-a cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), and scraped into PBS containing 10 mM HEPES buffer, pH 5.5 (unless otherwise indicated), and protease inhibitors (aprotinin, 20 μg/ml; benzamidine 5 mM). All subsequent manipulations were carried out at 4°C. Cells were homogenized with a glass-glass homogenizer; cellular breakage was assessed by loss of trypan blue exclusion. A postnuclear supernatant was prepared by centrifugation at $700 \times g$ for 10 min, and various additions (detergent, calcium ions) were made, as indicated in the text. After 10 min on ice, the supernatants were clarified by centrifugation at $10,000 \times g$ for 10 min, applied to 15–40% sucrose gradients, and subjected to centrifugation for 18 h in a swinging bucket rotor at $300,000 \times g$. Eleven fractions were collected beginning from the top of the gradient.

Immunoblotting Aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with specific antipeptide antisera as described [19]. The following antisera (all obtained from Dr. V. Hearing, National Cancer Institute) were utilized: αPEP7 (carboxy terminus of tyrosinase, 1:200), αPEP1 (carboxy terminus of TRP-1, 1:500), αPEP2 (amino terminus of TRP-1, 1:200), and αPEP8 (carboxy terminus of TRP-2, 1:500). A rat monoclonal antibody to lysosome-associated membrane protein-1 (LAMP-1) was employed as described previously [20].

Enzymatic Assays Dopachrome tautomerase was assayed spectrophotometrically as the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [21]. Dopachrome was prepared as described [22]. The tyrosine hydroxylase activity of tyrosinase was determined as described previously [23] by a radiometric assay adapted from that originally developed by Pomerantz [24]. Results are presented as the means of triplicate determinations.

Table II. Immunoprecipitation of "Complexed" Tyrosinase by αTRP1 and αTRP2[a]

	Tyrosinase Activity Immunoprecipitated (cpm)	Total Activity (%)
"Free" tyrosinase (24,392 cpm/assay)		
NRS	0	0
αTRP-1	1660	7
αTRP-2	528	2
"Complexed" tyrosinase (15,432 cpm/assay)		
NRS	0	0
αTRP-1	7561	49
αTRP-2	9097	59

[a] Peak I and Peak II from HPLC purification step shown in Table I, representing, respectively, the "complexed" and "free" tyrosinase peaks were pooled and the tyrosinase activity of each determined. Aliquots of each were subjected to immunoprecipitation by normal rabbit serum (NRS) or antiserum to TRP-1 (αPEP1) and αTRP-2 (αPEP8), and the immunoprecipitates resuspended and assayed for tyrosinase activity as described [21]. Results of triplicate determination are shown. The experiment was repeated twice with similar results.

RESULTS

When the dopachrome tautomerase activity of TRP-2 was purified as described previously [17,18] from detergent extracts of Cloudman melanoma cells, tyrosinase activity copurified with it through multiple steps (Table I). We have shown previously that upon gel-filtration HPLC, dopachrome tautomerase activity migrated in the void volume, corresponding to a Mr of at least 200 kD, along with approximately 20% of the total tyrosinase activity [18]. In contrast, 80% of tyrosinase activity migrated at a position in the column corresponding to its monomer Mr (~75 kD) [18]. This observation suggested the possibility of a high-molecular-weight complex between dopachrome tautomerase (TRP-2) and a portion of the total tyrosinase.

We reasoned that if the tyrosinase-related protein family existed as a complex, then antibodies to TRP-1 and TRP-2 should be able to immunoprecipitate tyrosinase activity from the high molecular weight gel filtration HPLC peak, but not from the peak representing uncomplexed "free" tyrosinase. The results in Table II demonstrate that antibodies to both TRP-1 and TRP-2 immunoprecipitated 49–59% of total tyrosinase activity present in "complex associated" tyrosinase, but only 2–7% of that from "free" tyrosinase.

When the peak of "free" tyrosinase was subjected to sucrose density gradient centrifugation, it migrated similarly to hemoglobin (native Mr 64 kD), with a lesser peak of ~200 kD (Fig 1*a*). In contrast, when the dopachrome tautomerase peak from gel filtration HPLC was similarly analyzed, the bulk of associated tyrosinase activity migrated as a broad peak of 200 to >700 kD (Fig 1*a*). TRP-2 migrated exclusively in a high-molecular-weight form, as expected from its behavior on molecular sieve HPLC (Fig 1*b, bottom*). No TRP-2 was detected in association with the peak of "free" tyrosinase from HPLC (Fig 1*b, top*).

Triton X-100 was used only in the initial phase of the purification scheme, and subsequent to the DEAE step all but those detergent molecules tightly bound to proteins would be removed by repeated washes. Thus, we asked whether the reintroduction of detergent would affect the possible association between tyrosinase, TRP-1, and TRP-2. Aliquots of the dopachrome tautomerase peak from gel filtration HPLC were treated with either Triton X-100 or CHAPS, and compared with untreated controls after sucrose density gradient centrifugation. The collected fractions were analyzed for tyrosinase, TRP-1, and TRP-2 by immunoblotting with specific antipeptide antisera [5–7]. The results in Fig 2 show that in the absence of added detergent, all three proteins were present, and sedimented towards the bottom of the gradient. The addition of CHAPS caused a slight shift to less dense fractions, corresponding to a decrease in Mr to the 200–400 kD range. In contrast, Triton X-100 treatment caused all three proteins to migrate in a fashion consistent with their monomeric (and thus uncomplexed) molecular weights. This sug-

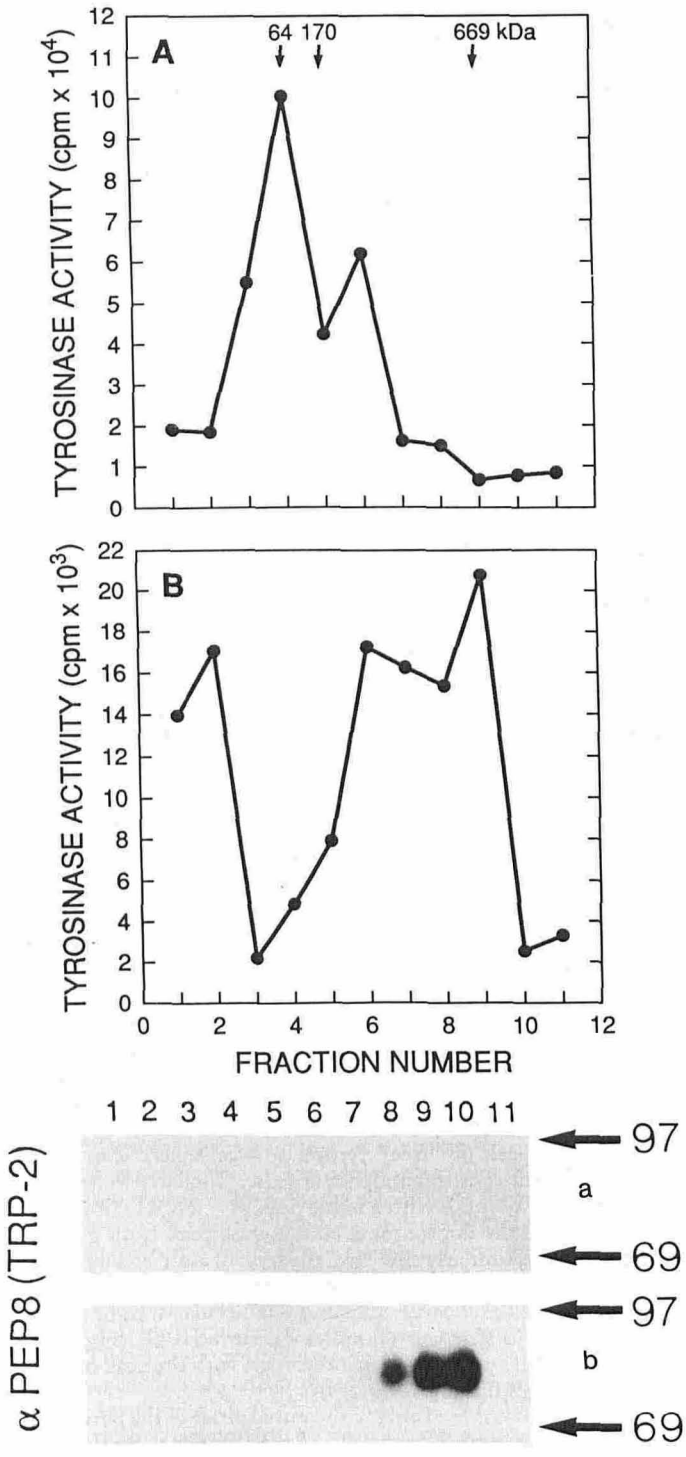

Figure 1. Sucrose density gradient: free versus complexed tyrosinase. *a)* Pooled HPLC fractions corresponding to *(A)* the low-molecular-weight peak devoid of dopachrome tautomerase activity but possessing tyrosinase activity and *(B)* the high-molecular-weight peak exhibiting both dopachrome tautomerase and tyrosinase activity, were subjected to sucrose density gradient centrifugation followed by determination of tyrosinase activity. The sedimentation behavior of proteins of known molecular weight is shown (64,000 = hemoglobin; 170,000 = human IgG; 669,000 = porcine thyroglobulin). The top of the gradient is at the *left,* the *bottom* (densest fractions) on the *right.* (The very-low-molecular-weight activity seen in fraction 1 *(B)* was not observed in subsequent experiments.) *b)* Pooled HPLC fractions were subjected to sucrose gradient analysis, but were analyzed instead by immunoblotting with an antiserum to the carboxy terminus of TRP-2. *Top)* Free tyrosinase HPLC peak; *bottom)* high molecular weight "complex" containing dopachrome tautomerase activity. The migration of bovine serum albumin (69,000 daltons) is shown.

gested that a metastable interaction existed between tyrosinase, TRP-1, and TRP-2, which could be disrupted by treatment with high concentrations of Triton X-100.

We also asked what effect dissociation of the complex by Triton X-100 would have on the activity of the associated tyrosinase. Table III demonstrates that exposure to Triton X-100 increased tyrosinase activity by approximately twofold.

Detergent Extraction The melanogenic complex was also observed in melan-a mouse melanocytes. When the postnuclear supernatant of melan-a cells was extracted with the detergent CHAPS, sucrose gradient density centrifugation followed by immunoblotting demonstrated that tyrosinase, TRP-1, and TRP-2 sedimented together in a broad, dense peak (Fig 3). Comparison with markers of known native molecular weight suggested that the size of these multimers was in the range of 200 → 700 kD. In contrast, substitution of Triton X-100 for CHAPS resulted in a disappearance of the high molecular weight form of each protein and the appearance of a peak that cosedimented with hemoglobin (64 kD), close to the monomer molecular weight of the members of the TRP family (~75 kD). When octylglucoside was used as the detergent, results intermediate between those obtained with CHAPS and Triton X-100 were obtained (not shown). We chose

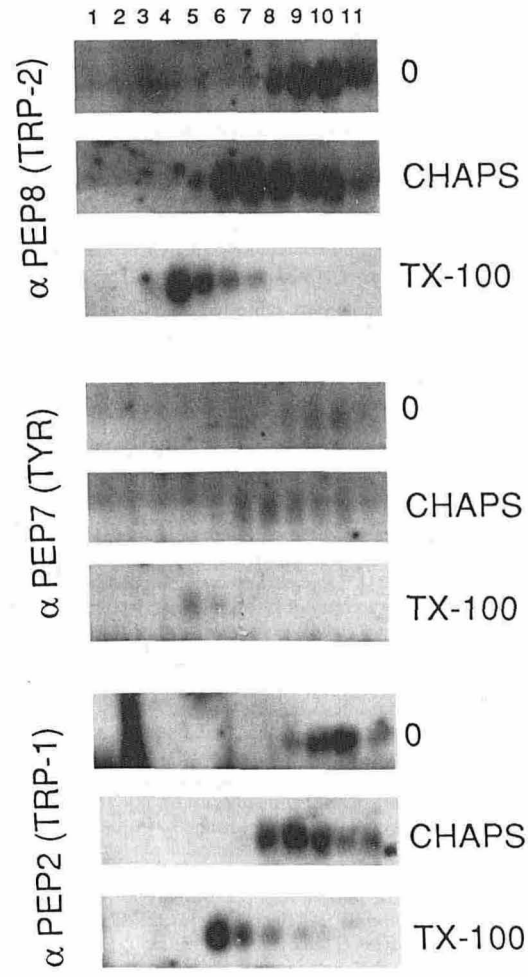

Figure 2. Effects of detergent on sedimentation behavior. The high-molecular-weight peak of DT/TRP-2 from HPLC was divided into three aliquots. Additions were made as follows: TX-100, Triton X-100 was added to a final concentration of 1% in the sample/0.1% in the gradient; CHAPS, CHAPS was added to a concentration of 2% in the sample/0.25% in the gradient, and 0, no detergent was added. Sucrose density sedimentation and analysis were as in Fig 1. In this and in subsequent figures the sedimentation of standard proteins was as follows: hemoglobin (64,000), fraction 4; IgG (170,000), fraction 5,6; thyroglobulin (669,000), fraction 10.

Table III. Effects of Triton X-100 on Tyrosinase Activity[a]

Tyrosinase Source	Tyrosinase Activity (cpm^3H_2O) (−) Triton X-100	(+) Triton X-100
HPLC-"high" mw	2445 ± 408	4655 ± 219
HPLC-"low" mw	10280 ± 791	11780 ± 393

[a] Tyrosinase was purified through the HPLC molecular sieve step as described in Table I. High and low molecular weight forms (see Fig 1) were assayed for tyrosinase activity at 37°C for 50 min in the absence (−) or presence (+) of Triton X-100 (1%, vol/vol). Results of tyrosinase activity represent averages of triplicates ± SD. The experiments were repeated twice, with similar results.

therefore to utilize CHAPS for subsequent studies on the TRP multimers. Although concentrations of 1–2% CHAPS were needed for efficient solubilization of the TRPs, lowering the CHAPS concentration in the gradient itself to ≤ 0.25% gave optimal results.

Detergents did not significantly alter the sedimentation behavior of LAMP-1, a type I membrane glycoprotein unrelated to the TRP family, which we have previously shown to be present in melanosomes [19,20].

Effects of Calcium, Ionic Strength, and pH Calcium is required for the maintenance of neuroendocrine secretory granule contents in aggregated form [25]. Melanosomes contain high concentrations of calcium [26], but the role of this cation in melanogenesis is not known. We thus sought to determine whether calcium was an important variable in the maintenance of the TRPs in a high molecular weight form. The results in Fig 4 demonstrate that in the presence of calcium (10 mM) the high molecular weight form of the TRPs was preserved. The inclusion of ethylenediamine tetraacetic acid (EDTA), which chelates a variety of divalent metal cations, including calcium, resulted in dissociation of the TRPs into monomers. Similarly, raising the ionic strength by inclusion of 100 mM potassium acetate or sodium chloride also favored dissociation of multimers.

TRPs in the multimeric complex appeared to be heavily glycosylated. This was best demonstrated in the case of TRP-2, because a substantial proportion of TRP-2 in cultured murine melanocytes and melanoma cells is present as the 68-kD partially glycosylated precursor [7,27]. As can be seen in Fig 4, this lower Mr form sediments preferentially as a monomer, in contrast to the mature glycosylated protein that sediments as a multimer. Similar results were obtained from analyses of TRPs from the eyes of 6–12-day-old mice [27,28].

We also investigated the effects of pH on the multimeric forms of the TRP family. We found that the presence of the TRPs in complexed form was dependent upon pH. The results shown in Fig 5 demonstrate that pH 5.5 was optimal for complex formation, although high molecular weight forms of all proteins could still be detected at pH 6.5.

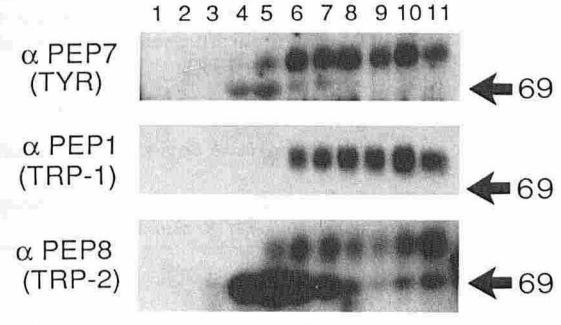

Figure 3. Density gradient analysis of melan-a cells. Melan-a cells were extracted with buffer containing 2% CHAPS, as described in *Materials and Methods,* and clarified extracts subjected to sucrose density gradient centrifugation and immunoblotting analysis. The migration of bovine serum albumin (69,000 daltons) and phosphorylase (97,000 daltons) is shown.

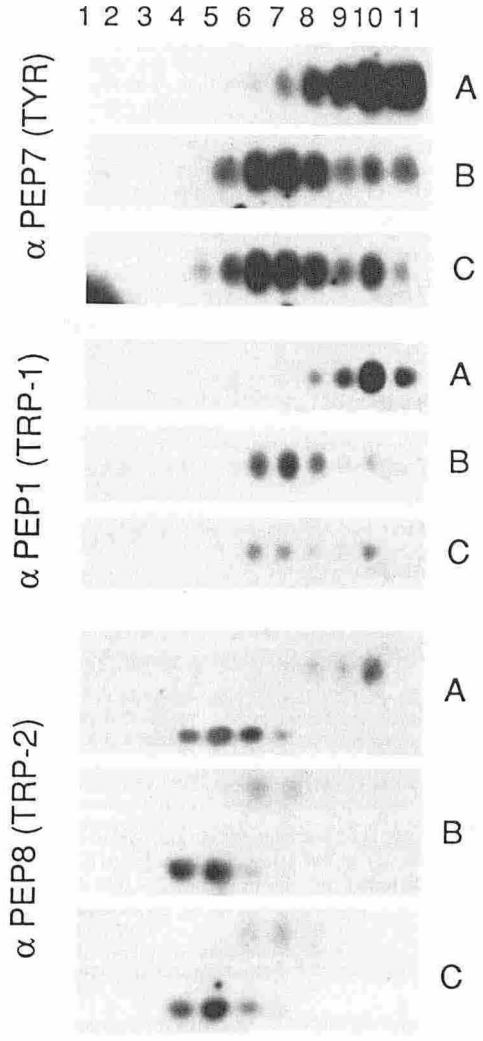

Figure 4. Effects of calcium and high ionic strength on the sedimentation behavior of the TRP family. Postnuclear supernatant from melan-a cells was divided into three equal aliquots and the content of ions adjusted as indicated below, followed by CHAPS solubilization and sucrose density gradient centrifugation and immunoblotting analysis of collected fractions. *A)* 10 mM $CaCl_2$, *B)* 3 mM EDTA, *C)* 100 mM KCl. Results similar to those in *(A)* were noted with 3 mM and 20 mM $CaCl_2$, as well.

DISCUSSION

We have demonstrated that each of the three members of the tyrosinase-related protein family — tyrosinase, TRP-1, and TRP-2 — is present in a high molecular weight form in cultured murine melanocytes. We present evidence that heteromultimers of the members of the TRP family can be detected, although we cannot rule out the presence of homomultimers as well. Other investigators, as well as ourselves, have detected the presence of tyrosinase, TRP-1, and TRP-2 in high molecular weight forms by gel filtration analysis [10–13,17,29] and by nondenaturing gel electrophoresis [31]. From previous analyses, it was suggested that the high molecular weight form of tyrosinase ("T4 isozyme") represented the mature glycosylated form of tyrosinase, which could associate with itself or with other proteins [10–13,29]. Our results support this interpretation in that the nonglycosylated precursors of the TRPs did not possess the same capacity to aggregate as did their mature glycosylated counterparts. However, our results employing Cloudman S91 melanoma cells from brown *(b/b)* mice with incompletely glycosylated TRP-1 suggest that the partial glycosylation the *b/b* TRP-1 undergoes [19,31] is sufficient to allow apparently normal multimerization to take place. We have obtained similar results when extracts of nontransformed melanocytes cultured from

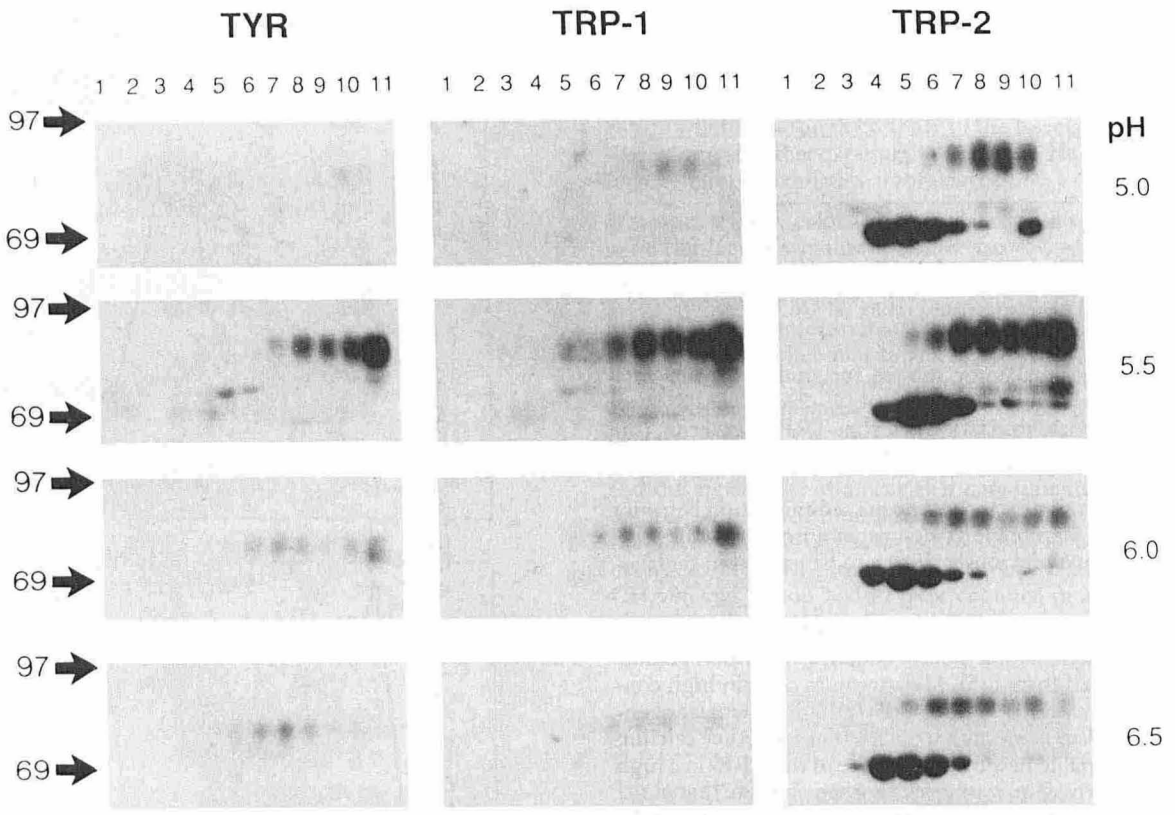

Figure 5. Effects of pH on complex formation. Melan-a cells were homogenized and extracted with CHAPS as described in *Materials and Methods,* except that the pH of the extraction buffer was varied from 5.0 to 6.5, followed by sucrose density gradient and immunoblotting analysis. The migration of bovine serum albumin (69,000 daltons) and phosphorylase (97,000 daltons) is shown.

mice homozygous for the *brown* mutation were analyzed (not shown).

Millimolar concentrations of calcium ions appear to favor preservation of the high-molecular-weight forms of the TRPs. Calcium has been detected in high concentrations within melanosomes [26], and it is known that this cation is important in the maintenance of neuroendocrine secretory granule proteins in an aggregated state [27]. The predicted amino acid sequence of tyrosinase and the TRPs each contain two potential copper-binding sites and two heme-binding sites, but common calcium-binding motifs are not evident.

The results of our study demonstrate that tyrosinase, TRP-1, and TRP-2 all interact to form a high-molecular-weight multimeric complex, and we have shown that the activity of tyrosinase is diminished in this complex when compared to "free" tyrosinase. These results are consistent with genetic evidence that indicates an interaction between the products of the *albino* and *brown* loci [32]. Coleman [33] observed that extracts of the skin of mice homozygous for the *brown* mutation, which is now recognized to result in the expression of a mutated TRP-1 [4], possess more than twice the melanin-synthesizing capacity of black skins when assayed *in vitro.* These results suggest that, as measured *in vitro,* TRP-1 may act as a modulator of tyrosinase activity, an interpretation with which our data are consistent.

Hearing and colleagues have presented evidence suggesting that TRP-1 protects tyrosinase from the denaturation that occurs upon incubation of the purified enzyme at room temperature or higher.† Although we observed that tyrosinase activity is diminished in the presence of TRP-1 and TRP-2, we did not test for the effects of complex formation on tyrosinase stability. It is possible, for example, that tyrosinase in complex form exhibits less activity but prolonged stability when compared with tyrosinase not associated in a complex.

Finally, the pH dependence of complex formation is especially deserving of comment. We and others have demonstrated that melanosomes share features in common with the lysosomal/endosomal lineage of organelles [19,20,34,35]. The intramelanosomal pH has been estimated to be as low as 3.0–5.0 [36], and exposure of tyrosinase to low pH can alter the activity of the enzyme [37,38]. The quality and polymerization of melanin is also pH sensitive [39]. The results of the current study suggest that the interaction of the members of the TRP family with one another would be favored by the low pH within the mature, melanizing melanosome. Thus, pH may play a critical role in regulating the process of melanogenesis.

Note Added in Proof: A high-molecular-weight form of tyrosinase has recently been described by Rieber MS, Rieber M: Specific tyrosinases associated with melanoma replicative senescence and melanogenesis. *Cancer Res* 53:2469–2471, 1993.

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