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Mutation in and Lack of Expression of Tyrosinase-Related Protein-1 (TRP-1) in Melanocytes from an Individual with Brown Oculocutaneous Albinism: A New Subtype of Albinism Classified as "OCA3"

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Summary

Most types of human oculocutaneous albinism (OCA) result from mutations in the gene for tyrosinase (OCA1) or the P protein (OCA2), although other types of OCA have been described but have not been mapped to specific loci. Melanocytes were cultured from an African-American with OCA, who exhibited the phenotype of Brown OCA, and his normal fraternal twin. Melanocytes cultured from the patient with OCA and the normal twin appeared brown versus black, respectively. Melanocytes from both the patient with OCA and the normal twin demonstrated equal amounts of NP-40-soluble melanin; however, melanocytes from the patient with OCA contained only 7% of the amount of insoluble melanin found from the normal twin. Tyrosinase-related protein-1 (TRP-1) was not detected in the OCA melanocytes by use of various anti-TRP-1 probes. Furthermore, transcripts for TRP-1 were absent in cultured OCA melanocytes. The affected twin was homozygous for a single-bp deletion in exon 6, removing an A in codon 368 and leading to a premature stop at codon 384. Tyrosine hydroxylase activity of the OCA melanocytes was comparable to controls when assayed in cell lysates but was only 30% of controls when assayed in intact cells. We conclude that this mutation of the human TRP-1 gene affects its interaction with tyrosinase, resulting in dysregulation of tyrosinase activity, promotes the synthesis of brown versus black melanin, and is responsible for a third genetic type of OCA in humans, which we classify as "OCA3."

Introduction

Human oculocutaneous albinism (OCA) is clinically heterogeneous, and the different types are best distinguished by the responsible gene involved. To date, two OCA genes have been identified, including the tyrosinase gene of OCA1 (tyrosinase-related OCA) (Tomita et al. 1989; Peltz et al. 1990; Chintamaneni et al. 1991*a*; Giebel et al. 1991*a*, 1991*b*) and the P gene of OCA2 (P-related OCA) (Gardner et al. 1992; Ramsay et al. 1992; Rinchik et al. 1993; Durham-Pierre et al. 1994; Lee et al. 1994). The genes responsible for other types of OCA remain unknown.

One unclassified type of albinism is Brown OCA, which exhibits, in Negro individuals, light brown skin, light brown hair, and blue to brown irides associated with nystagmus and reduced visual acuity (for review see King et al. [1994]). Brown OCA was originally identified in affected individuals of Nigeria (King et al. 1980) and subsequently within African-Americans (King et al. 1985). Brown OCA is phenotypically distinct from OCA1 and genetically distinct from OCA2, and family studies show autosomal recessive inheritance (King et al. 1980; King and Rich 1986). It is interesting to note that the phenotype of moderate reduction in oculocutaneous pigmentation as found in African or African-American individuals with Brown OCA has not yet been reported in Caucasian or Mongoloid individuals.

Mutant alleles at the autosomal *brown* locus in mice result in the dilution of black pelage coloration to brown (Silvers 1979) in a pattern similar to human Brown OCA. The gene at the *brown* locus encodes the tyrosinase-related protein-1 (TRP-1), a recently identified melanocyte specific gene product with ~40% amino acid–sequence homology to tyrosinase (Shibahara et al. 1986; Jackson 1988). The human TRP-1 gene has been mapped to human chromosome 9p23 (Chintamaneni et al. 1991*b*; Murty et al. 1992), and the protein has been characterized (Jiménez et al. 1991; Urquhart 1991; Hearing et al. 1992). The function(s) of TRP-1 is in dispute; however, numerous catalytic activities have been suggested (Halaban and Moellmann 1990; Jiménez et al. 1991; Hearing 1993; Jiménez-Cervantes et al. 1993; Winder et al. 1993). It has only recently been demonstrated that TRP-1 in mice exhibits a novel

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DHICA oxidase activity (Jiménez-Cervantes et al. 1994; Kobayashi et al. 1994). In addition, cultured melanocytes from brown mutant mice transfected with the TRP-1 cDNA resulted in the production of black/dark brown melanin (Bennett et al. 1990), which verifies that TRP-1 is a key regulatory protein in the production of black, rather than brown, melanin. In mice, TRP-1 also serves an important function in stabilizing tyrosinase and other melanosomal enzyme activities (Winder et al. 1994).

In this report, we describe properties of cultured melanocytes established from a set of fraternal African-American twins of whom one exhibited Brown OCA. DNA isolated from cultured fibroblasts of the individual with Brown OCA exhibit a point mutation in exon 6 of the TRP-1 gene, and cultured melanocytes do not express transcripts or products from that locus. In addition, these melanocytes produce predominantly brown soluble melanin and exhibit muted tyrosine hydroxylase activity within the intact cell. We conclude that this patient represents a third genetically distinct form of oculocutaneous albinism and should be classified as "OCA3."

Patients, Material, and Methods

Patients and Melanocyte Cultures

DZ twin males were evaluated at 1 d of age (fig. 1*a*). One twin (the proband) had light brown skin and hair and blue/gray irides with a red reflex, consistent with Brown OCA (King et al. 1980). The unaffected twin had dark hair and skin pigment that was typical for a normally pigmented African-American newborn male. The twin with Brown OCA developed bilateral nystagmus by the age of 1 year. Family history indicated that other family members (a sibling and the maternal grandmother) had been born with hypopigmentation similar to the affected twin that is associated with an increase in pigmentation with age, consistent with Brown OCA. Unfortunately, additional information or tissue samples from this subject family are impossible to acquire.

Foreskins were obtained during routine circumcision from both twins and were used to develop melanocyte cultures as described by Boissy et al. (1991) and approved of by the Institutional Review Board of the University of Cincinnati. Foreskins or punch biopsies were also obtained and used to develop melanocyte cultures from normal African-American and Caucasian donors and from a donor with OCA1 having no pigment, and a donor with OCA2 having a classic tyrosinase-positive OCA phenotype (King et al. 1994). Established cultures of melanocytes were processed for electron microscopy and for dihydroxyphenylalanine (DOPA) histochemistry as described by Boissy et al. (1991).

Antibodies

Antibodies—corresponding antigens (and source) that were used for immunofluorescence and/or immunoblotting throughout this report and exhibit cross-reactivity with human proteins—are as follows: TA99—human TRP-1 (provided by A. Houghton and S. Vijayasaradhi [Thomson et al. 1985]); α-SL—avian TRP-1 (provided by R. Boissy [Austin and Boissy 1995]); TMH-1—murine TRP-1 (provided by V. Hearing [Tomita et al. 1985]); αPEP-1—carboxy terminus of murine TRP-1 (provided by V. Hearing [Jiménez et al. 1989]); αPEP-2—amino terminus of murine TRP-1 (provided by V. Hearing [Jiménez et al. 1989]); αPEP-5—amino terminus of murine tyrosinase (provided by V. Hearing [Jiménez et al. 1991]); αPEP-7—carboxy terminus of murine tyrosinase (provided by V. Hearing [Jiménez et al. 1991]); αTy-SP—hamster tyrosinase (provided by S. Pomerantz [Halaban et al. 1983]); αPEP-8—murine DOPAchrome tautomerase (provided by V. Hearing [Tsukamoto et al. 1992]); αS-100—bovine S-100 (Dako Corp. [Cochran et al. 1982]); αKD40—hu-

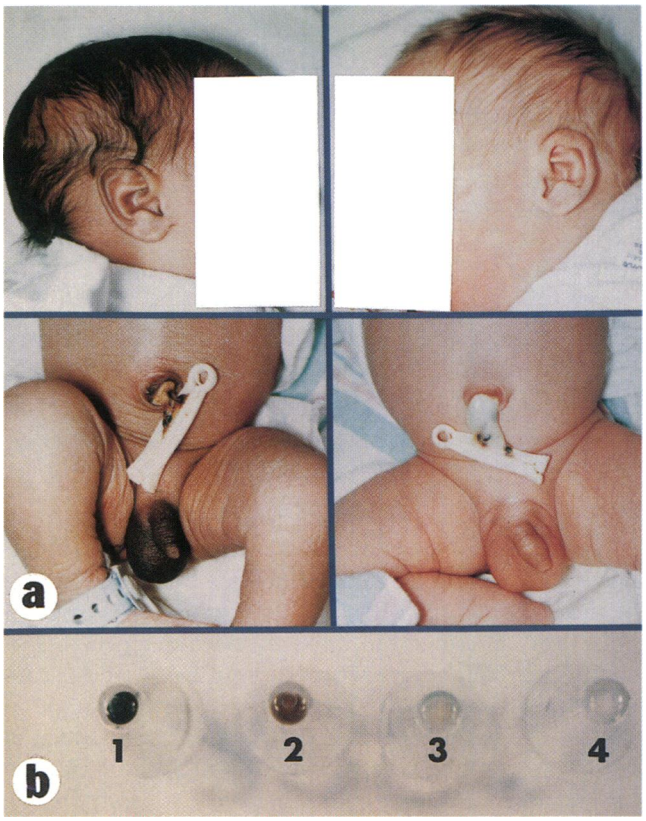

Figure 1 Phenotype of the twins and pigmentation of their cultured melanocytes. *a*, Proband with Brown OCA (*upper right column*) and his unaffected normal twin brother (*upper left column*) from an African-American family. The affected twin presented with light-brown skin and hair pigmentation characteristic of Brown OCA. Foreskins from these fraternal twins were used to establish melanocyte cultures as described in Patients, Material, and Methods. *b*, Melanin color synthesized by melanocytes in culture resembling the phenotype of the donor. Pellets of two million melanocytes cultured from (1) the unaffected normal twin are black, (2) the affected proband are brown, (3) a Caucasian neonate are dusty gray, and (4) a tyrosinase-negative OCA1 neonate are amelanotic.

man granulophysin (provided by J. Gerrard [Gerrard et al. 1991]); αMPR—human mannose-6-phosphate receptor (provided by W. Sly and J. Grubb [Oshima et al. 1988]); α-DNP—dinitrophenol (Oxford Inc. [Anderson et al. 1984]); and αTNF-α—tumor necrosis factor-α (Immunex Research).

Assays

Insoluble melanin was assayed with lysates of cultured melanocytes solubilized in 0.5% NP40/PBS, and soluble melanin was assayed within lysates further solubilized in 0.1 N NaOH as described elsewhere (Zhao and Boissy 1994). Tyrosine-hydroxylase activity was assayed with cultured melanocytes by the in situ (i.e., using intact cells), the in vitro (i.e., using cell lysates), and the post-fixation (i.e., using half-strength Karnovsky's fixative) methods as described elsewhere (Zhao and Boissy 1994) Indirect fluorescence immunocytochemistry and identification of acidic organelles were performed on cultured melanocytes, fixed in 5% formalin, and permeabilized with 100% methanol as described elsewhere (Zhao et al. 1994*a*).

Proteins were identified in cultured melanocytes solubilized in 0.5% NP40/PBS by use of Western immunoblotting as described elsewhere (Zhao and Boissy 1994). Transcripts for TRP-1 and tyrosinase were assayed in cultured melanocytes by use of Northern blot analysis as described elsewhere (Austin and Boissy 1995). The cDNA probes used for Northern blot analysis were: human TRP-1 (gp75) cDNA obtained from S. Vijayasaradhi, human tyrosinase (TY) (Bouchard et al. 1989,) and human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) from Clonetech Laboratories. The Image Quant analysis software was used to determine the relative levels of ^{32}P signal for each message. For comparison, the levels were measured as "peak" values in "pixel" units. For Southern blot analysis, isolated genomic DNA was digested with either *Eco*R1, *Hin*dIII, or *Hae*III, separated on a 0.7% agarose gel, and transferred to nitrocellulose by use of standard techniques (Southern 1975). The transferred DNA was hybridized to the TRP-1 cDNA probe described above, which was radioactively labeled with random-primer labeling an [$\alpha^{32}P$]-dCTP (Feinberg and Vogelstein 1983). Hybridized bands were visualized with autoradiography.

PCR Amplification of Genomic DNA/SSCP Analysis/ DNA Sequencing

The human TRP-1 cDNA sequence has been published previously (Cohen et al. 1990; Urquhart 1991). The exons that make up the coding region of the TRP-1 gene were amplified for SSCP analysis with the following primer pairs by use of methods established in our laboratory (Oetting et al. 1994). Exon 2: 5′-GTGCTTCAGTCTTCTCTACA-3′, 5′-TATGAGAACCCTCTGGTCAC-3′; Exon 3: 5′-TCAGGAGAAATCTTCTGGACT-3′, 5′-TGCATGTCTTTCTCCAGACG-3′; Exon 4:5′-GAAATGTTGCAAGAGCCTTCTT-3′, 5′-GTTACAAAGTGTTCCCAGGG-3′; Exon 5: 5′-GCACCGAGGATGGGCCAATT-3′, 5′-CTTCCACTGTGTTTCGGAAAC-3′; Exon 6: 5′-GTTACAGTGACCCCACGGGAA-3′, 5′-CAGCATTGTATCTCCTCAGCC-3′; Exon 7: 5′-ATATATCCACATTTCCATTGGA-3′, 5′-TTGGCCATTGAATTTCATAAG-3′; and Exon 8: 5′-GTCGGGAGTTTAGTGTACCT-3′, 5′-TCTGAAAGGGTCTTCCCAGC-3′

These amplifications were carried out in 50-μl reactions containing 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton-X100, 200 μM of each dNTP, 5 pmol of each primer, 1.25 U *Taq* DNA polymerase (Promega), and 100–200 ng of genomic DNA isolated from fibroblasts cultured from the two subjects. Following a 4-min denaturation step, reactions were cycled 35 times under the following conditions: 95°C, 1 min; T_m (5°C), 1 min; 72°C, 1.5 min. This was followed by a final 10-min extension period at 72°C. The amplification products were then checked on an agarose gel stained with ethidium bromide. After amplification, 6 μl of the exon product were mixed with a formamide-based loading dye, denatured at 95°C for 5 min, and snap-cooled on ice (Oetting et al. 1994). These samples were then loaded onto 8% polyacrylamide, nondenaturing gels and run at 4°C and 100 v for 16–18 h. Bands were visualized by silver staining with the following procedure: 10 min in 10% ethanol, 3 min in 1% HNO_3, 1.5 h in Silver Stain Reagent (Bio-Rad), rinse with H_2O, develop with Bio-Rad silver-stain developer, and 5 min in 5% acetic acid to stop development. Exons that displayed mobility shifts on SSCP analysis were sequenced by use of autoradiography to identify mutations (Oetting et al. 1994). Sequencing was carried out with the primers listed above. Primers were end labeled with γ-^{32}P-ATP, and sequencing reactions were carried out with the CircumVent™ cycle sequencing kit from New England Biolabs. Products of the sequencing reactions were separated on an 8% acrylamide, 8 M urea sequencing

Table 1

Quantitation of Melanin in Cultured Human Melanocytes

	Melanocyte Culture Line			
Melanin[a]	Normal Twin	Brown OCA Twin	Caucasian Control	OCA1 Control
Supernatant	119.8	116.6	14.9	0
Pellet	1,177.8	66.4	35.1	0
Total	1,307.6	183.0	50.0	0

[a] Melanin was determined in established cultures of melanocytes from the normal pigmented twin, the proband with Brown OCA, a Caucasian, and an OCA1 neonate, as described in Patients, Material, and Methods.

gel by use of a Bio-Rad Sequi-Gen™ sequencing apparatus run at 70 w for ~2 h.

Results

Pigmentation

Cultured melanocytes were pelleted by centrifugation. The melanocytes from the Brown OCA twin displayed a brown color, in contrast to the black color of the melanocytes from the normal twin, the dusty gray color of control Caucasian melanocytes, and the white color of OCA1 (tyrosinase-related OCA) melanocytes (fig. 1*b*). Melanin contents were determined within the supernatants and the pellets of melanocyte lysates solubilized by NP40 (i.e., soluble vs. insoluble melanin, respectively) (table 1). Melanocytes from the twin with Brown OCA and the normal twin exhibited similar amounts of soluble melanin in the supernatants; however, there was an ~93% reduction in amount of insoluble melanin in the pellet from the Brown OCA melanocytes compared to those from the unaffected twin.

Ultrastructure of Cultured Melanocytes

Electron microscopic analysis (fig. 2) of cultured melanocytes from the twin with Brown OCA demonstrated only morphologically normal early melanosomes (i.e., stage I and stage II). In contrast, melanocytes cultured from normally pigmented African-American individuals, including the unaffected twin, contained numerous fully matured and pigmented stage IV melanosomes.

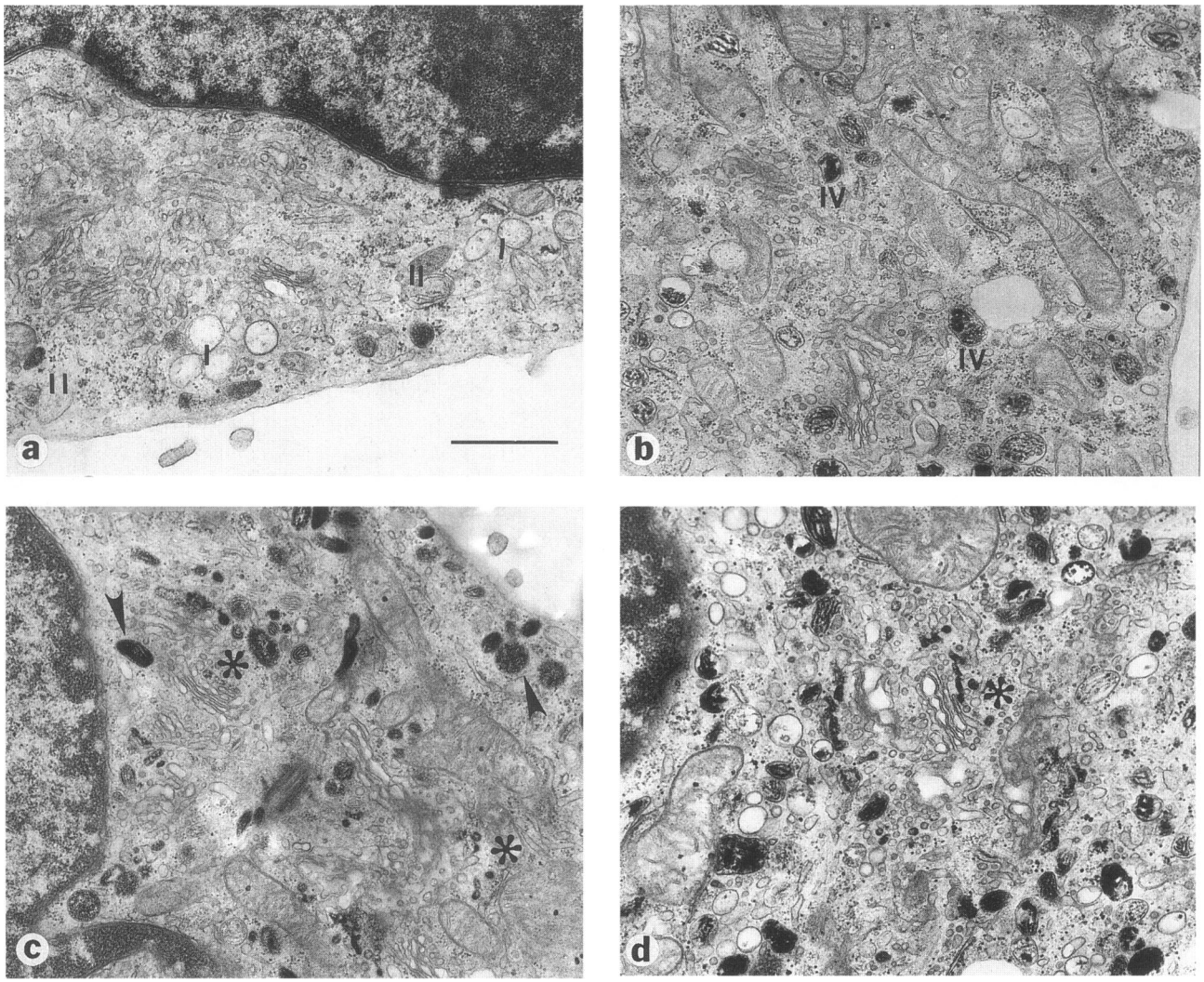

Figure 2 Ultrastructure and DOPA histochemistry of cultured melanocytes. *a,* Cultured melanocytes established from the twin with Brown OCA, exhibiting only stage 1 premelanosomes (I) and minimally pigmented stage II/III melanosomes (II). *b,* Cultured melanocytes established from the normally pigmented twin exhibit all stages of melanosomes, which prominently consist of heavily pigmented stage IV melanosomes (IV). After DOPA histochemistry, melanocytes from the twin with Brown OCA (*c*) exhibit extensive reaction product within many of the melanosomes (arrowheads) and at the trans-Golgi network (*) identical to the reaction profile in the melanocytes from the normal twin (*d*). Bar = 1.0 μ.

After DOPA histochemistry, the induced melanin reaction products accumulated normally in the Brown OCA melanosomes and trans-Golgi network, comparable to those from the unaffected twin's melanocytes.

Immunolocalizations

Cultured melanocytes from the twin with Brown OCA and the normal twin, as well as from a battery of melanocytes from normally pigmented Caucasian and African-American individuals and individuals with OCA1 and OCA2, were screened by indirect immunofluorescence microscopy for TRP-1 (using PEP-1, TA99, αSL, and TMH-1 antibodies), S-100 antigen, granulophysin (using αKD-40 antibody), acidic organelles, and tumor necrosis factor-α. The results demonstrated that melanocytes from the twin with Brown OCA lacked exclusively all reactivity to the TRP-1–specific antibodies (fig. 3, table 2).

We also screened extracts of these cultured melanocytes by western immunoblotting analysis for TRP-1 (using PEP-1, PEP-2, TA99, and αSL antibodies), tyrosinase (using PEP-5, PEP-7, and αTY-SP), DOPAchrome tautomerase (using PEP-8), granulophysin (using αKD-40), and the mannose-6-phosphate receptor. Again, reactivities were comparable in both lines, except that melanocytes from the twin with Brown OCA exclusively lacked immunoreactivity against all the TRP-1 specific antibodies (fig. 4, table 2). In addition, DOPA staining demonstrated DOPA oxidase activity in melanocytes from both the twin with Brown OCA and the normal twin (fig. 4).

Northern and Southern Analysis

Total RNA was extracted from the two twin melanocyte cell lines, plus an additional culture of normal human Caucasian melanocytes, and probed by Northern blot analysis for TRP-1, tyrosinase (TY), and glyceraldehyde-3 phosphate dehydrogenase (G3PDH) transcripts sequentially (fig. 5). Transcription of tyrosinase and G3PDH were comparable in all lines, but the melanocytes from the twin with Brown OCA exhibited an exclusive and total lack of TRP-1 transcript. In addition, genomic DNA was isolated from cultured fibroblast lines developed from the two twins, digested with either *Eco*R1, *Hin*dIII or *Hae*III and probed by Southern blot analysis using the TRP-1 cDNA. The resulting band patterns for each restriction enzyme tested did not differ between the two individuals (data not shown).

Genomic Analysis

Using PCR amplification of each exon coupled with SSCP analysis and direct DNA sequencing, we found the affected twin to be homozygous for a single–base pair deletion in exon 6 (fig. 6). The deletion removes an A in codon 368 leading to a premature stop at codon 384. SSCP analysis, as well as sequence analysis, showed no alterations in the other exons.

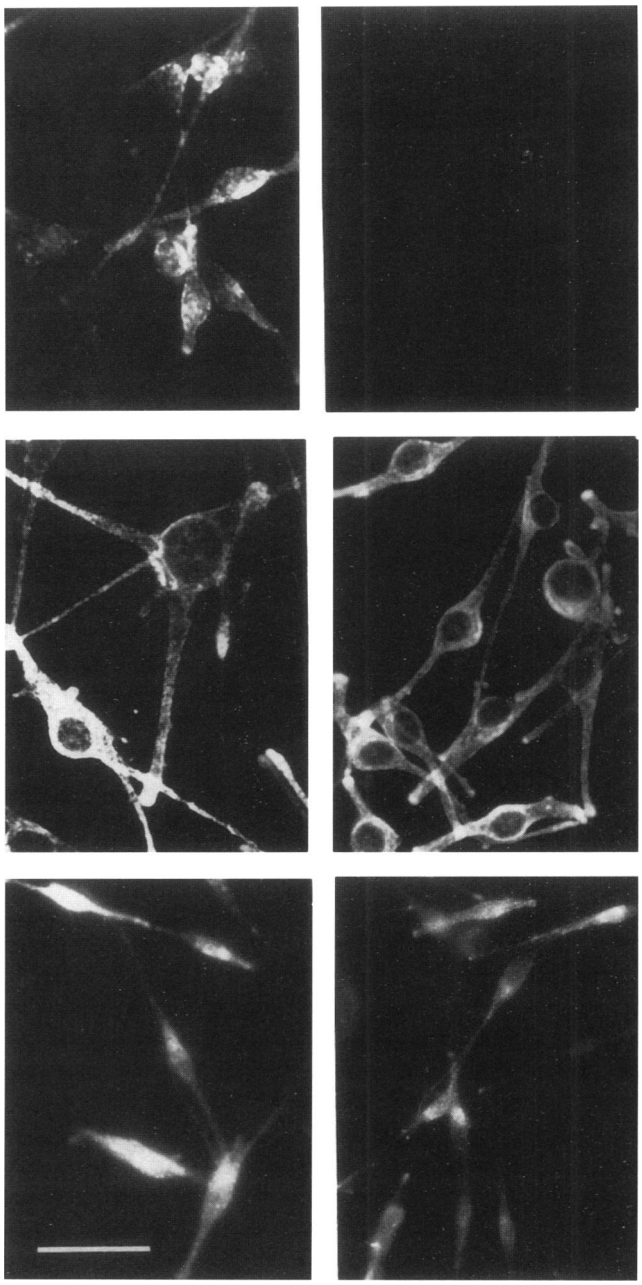

Figure 3 Expression of TRP-1, granulophysin, and acidic organelles in cultured melanocytes. Melanocytes derived from the twin with Brown OCA (*right column*) and the normal twin (*left column*) were stained by indirect immunofluorescence for TRP-1, by using the TA99 monoclonal antibody (*top row*), granulophysin (*middle row*), and acidic organelles (*bottom row*). The pattern of immunolocalization for all the antigens appeared granular throughout the cell body and dendrites with a perinuclear density of stain at the Golgi zone, except for TRP-1, which was completely absent in the melanocytes from the OCA twin. Bar = 40.0 μ.

Tyrosinase Assay

The tyrosine hydroxylase activities of the cultured melanocytes from the twin with Brown OCA, the normal twin, and individuals with OCA1 or OCA2 were

Table 2

Summary of Results from Immunoprobing and DOPA Staining of Cultured Melanocytes

Experimental Protocol[a]	Protein Detected	Brown OCA Twin	Normal Twin
Indirect immunofluorescence:			
αPEP-1	TRP-1 (murine)	−	+
TA99	TRP-1 (human)	−	+
αSL	TRP-1 (avian)	−	+
TMH-1	TRP-1 (murine)	−	+
αS-100	S-100 (bovine)	+	+
αKD-40	Granulophysin	+	+
αDNP	Dinitrophenol	+	+
αTNF-α	TNF	−	−
Western immunoblotting:			
TA99	TRP-1 (human)	−	+
αPEP-1	TRP-1 (murine)	−	+
αPEP-2	TRP-1 (murine)	−	+
α-SL	TRP-1 (avian)	−	+
αPEP-8	DOPAchrome tautomerase (murine)	+	+
αTy-SP	Tyrosinase (hamster)	+	+
αPEP-5	Tyrosinase (murine)	+	+
αPEP-7	Tyrosinase (murine)	+	+
αKD-40	Granulophysin	+	+
α-M6PR	Mannose-6-phosphate receptor	+	+
DOPA histochemistry	...	+	+

[a] Indirect immunofluorescence, western immunoblotting, and DOPA histochemistry were performed on cells and/or extracts of melanocytes cultured from the brown OCA and the pigmented twins using the antibodies listed in the table as described in Patients, Material, and Methods.

assayed by three methods that measured activities in (*a*) cellular lysates (in vitro assay), (*b*) intact/live cells (in situ assay), or (*c*) intact/fixed cells (postfixation assay), respectively (table 3). The tyrosine hydroxylase activities for melanocyte lysates were comparable between the twin with Brown OCA, the normal twin, and an unrelated individual with OCA2 whose melanocytes expressed normal amounts of TRP-1. In marked contrast, the tyrosine hydroxylase activities for intact/live cells of the twin with Brown OCA and the individual with OCA2 were ~30% of the value for the normal twin. After fixation of the cells in the culture dish, the discrepancy in tyrosine hydroxylase activities between the two twins was not corrected, in contrast to the discrepancy between the activities of the OCA2 individual and the normal twin, which was corrected. With all these assays, melanocytes from the OCA1 individual exhibited no tyrosine hydroxylase activity.

The in situ assay reported above was performed with the addition of 1 μCi/ml of ^{3}H-tyrosine only to the melanocyte growth medium. However, when 80 μmol/L of L-DOPA was also added to the melanocyte growth medium prior to the in situ assay, the reduction in tyrosine hydroxylase activity for the melanocytes cultured from the twin with Brown OCA was normalized to the value of the melanocytes from the control twin (fig. 7).

Discussion

OCA was originally categorized into two forms, i.e., tyrosinase negative and tyrosinase positive (Witkop 1971; King and Witkop 1976). Tyrosinase-negative OCA individuals were those who had a complete lack of melanin pigment and whose anagen hair bulbs, when incubated in a histochemical solution containing tyrosine, or DOPA, produced no melanin reaction product. In contrast, tyrosinase-positive OCA individuals were those who exhibited some pigmentation phenotypically and demonstrated a DOPA-positive hair bulb test. However, as the genes responsible for different types of OCA have been identified, the descriptive terms have subsequently changed and have been redefined (King et al. 1994). OCA1 refers to those types of OCA that result from mutations of the tyrosinase gene on chromosome 11q14-21 (Barton et al. 1988; Tomita et al. 1989; Giebel et al. 1991*a*, 1991*b*; King et al. 1991), which now includes nonpigmenting and pigmenting types (i.e., yellow OCA and minimal pigmented OCA). Mutations of the P gene on chromosome 15q11.2-13, the human homologue of the murine pink-eyed dilution locus (Gardner et al. 1992), has been identified in a large percentage of individuals with the classic tyrosinase-positive form of OCA (Ramsay et al. 1992; Rinchik et al. 1993; Durham-Pierre et al. 1994; Lee et al. 1994). This genetically defined form of OCA has been

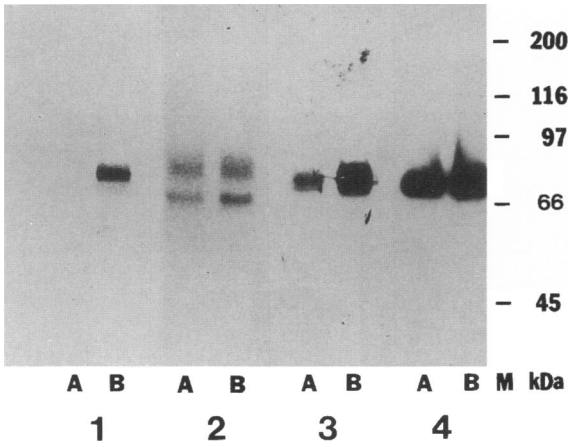

Figure 4 Immunoblot analysis and DOPA reactivity of melanocyte lysates. Proteins in cellular extracts of established melanocyte cultures derived from the twin with Brown OCA (lanes A) and the normal twin (lanes B) were separated by SDS/PAGE and either stained immediately with l-DOPA (group 4) or transferred to nitrocellulose and immunoprobed for TRP-1 by using αPEP-1 (group 1), DOPAchrome tautomerase by using αPEP-8 (group 2), and tyrosinase by using αTy-SP (group 3). Banding patterns between the two melanocyte lines were similar for each melanocyte-specific protein, except for TRP-1, which was absent in melanocytes from the twin with Brown OCA.

labeled "OCA2" (King et al. 1994). We now present critical observations in this report that a third genetically distinct form of OCA, which presents as Brown OCA, expresses a molecular and biochemical abnormality in tyrosinase-related protein-1 (TRP-1). We propose that this form of albinism results from mutations of the TRP-1 gene on chromosome 9p23 (Murty et al. 1992) and suggest that the correct nosology would be OCA3 for this type of albinism.

In this report, we demonstrate for the first time that an African-American OCA patient, whose skin and hair pigmentation is light brown as opposed to black, expresses a germ-line mutation in the TRP-1 gene and exhibits a lack of TRP-1 message, protein, and putative function. This case represents a human homologue to mutations at the murine Brown TRP-1 locus, which results in a switch from black to brown pelage coloration (Jackson 1988; Jackson et al. 1990). In mice, there are six alleles at the *brown* locus that have been analyzed by molecular techniques (Jackson et al. 1990; Jackson 1994). The *light* (B^{lt}) and *brown* (b) alleles express point mutations; the *cordovan* (b^{c}) and *cordovan-Harwell* (b^{cH}) alleles express dramatically reduced levels of TRP-1 transcripts and the *White-based brown* (B^{w}) and the *brown-Pasteur* (b^{Pas}) express no TRP-1 mRNA. It is interesting to note that the phenotype of *cordovan-Harwell* is somewhat darker than either *brown* or *brown-Pasteur* (Jackson 1994), which suggests that reduced amounts of TRP-1 impeded black eumelanin synthesis less extensively than either a dysfunctional TRP-1 or no TRP-1, respectively. This correlates with the dramatic hypopigmentation of the proband expressing no TRP-1 in this report. However, how other mutations affecting function and/or expression levels of TRP-1 influence cutaneous pigmentation in humans needs to be ascertained.

The phenotype of this patient resembles the minimal depigmentation characteristic of the type of oculocutaneous albinism called "Brown OCA," which in the literature has been described only in people of Negroid heritage (King et al. 1985). It now needs to be determined whether all forms of Brown OCA in humans result from mutations affecting the presence or function of TRP-1. However, alternatively, some types of OCA with a brown phenotype may result from mutations affecting the function of melanocyte-specific gene products other than TRP-1. Potential loci, which would include those for DOPAchrome tautomerase (TRP-2) (Jackson et al. 1992) and the MSH receptor, have not been associated with a human clinical condition. In addition, the phenotypic consequence of mutations affecting TRP-1 in Caucasian and Mongoloid races needs to be ascertained. That Brown OCA has thus far been observed only in individuals with darker complexion might be due simply to our inability to detect the phenotype in individuals with lighter European complexions (skin types 1, 2, and 3) (King et al. 1980, 1985).

We demonstrated that the melanocytes cultured from the twin with Brown OCA lack both TRP-1 and its RNA message. The mutation in the TRP-1 gene identified in this Brown OCA patient leads to premature termination of transcription in exon 6, which would putatively result in a slightly truncated TRP-1 molecule. However, we have identified no transcript or protein in these melanocytes suggesting that the aberrant transcript is highly unstable. It has previously been demonstrated that nonsense mutations that produce a premature termination codon can dramatically decrease the stability of the associated mRNA (Peltz et al. 1990; Surdej et al. 1994). Specific examples in human diseases consist of Duchenne or Becker muscular dystrophy (Chelly et al. 1990) and triosephosphate isomerase deficiency (Daar and Maquant 1988). In addition, specific cellular destabilizing pathways that function to degrade mRNAs containing nonsense codons have been described (Leeds et al. 1991; Pulak and Anderson 1993). A similar situation has been reported for the murine microphthalmia locus (Steingrimsson et al. 1994). One of the ~17 mutant alleles at this locus, mi^{ce}, expresses a transition from a C to T at bp 916 that introduces a stop codon after residue 262 of the 419–amino acid basic helix-loop-helix-leucine zipper (bHLH-ZIP) protein. These mi^{ce} transcripts are barely detectable in Northern analysis of mutant tissue (Steingrimsson et al. 1994), and the authors suggested that this mutation thus appears to affect mi mRNA stability.

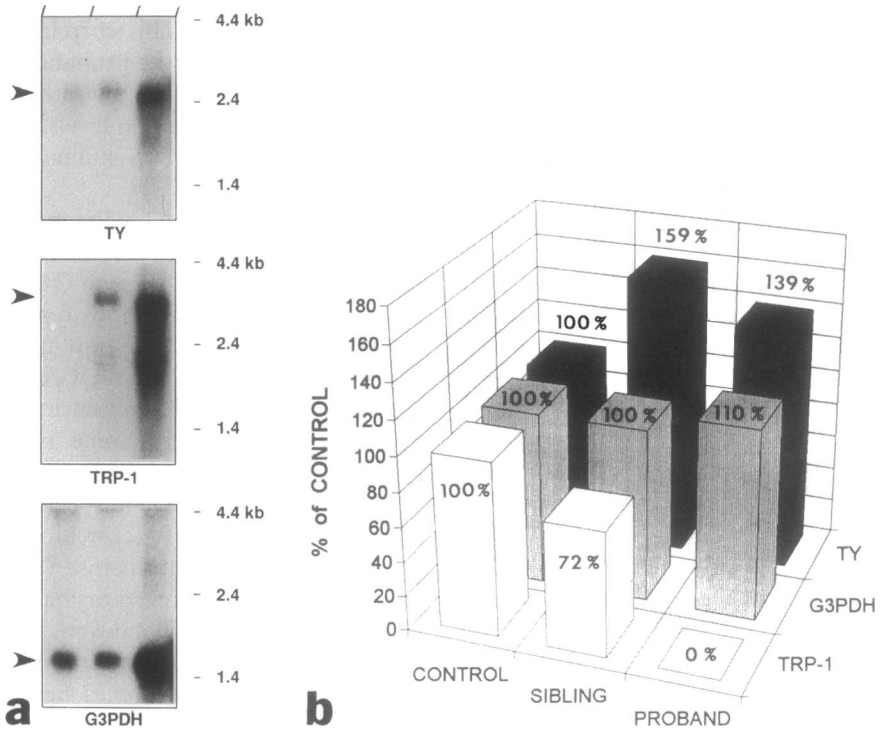

Figure 5 Northern blot analysis of cultured melanocytes. *a,* Total RNA in cultured melanocytes derived from the twin with Brown OCA (*left lane*), the normal twin (*middle lane*), and an unrelated Caucasian donor (*right lane*), hybridized with labeled cDNA probes for tyrosinase (TY panel), TRP-1 (TRP-1 panel), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH panel). (Five times more RNA was loaded onto the right lane than was loaded onto the left and middle lanes.) Normal transcript sizes were identified (arrowheads) among the three groups tested, except for the twin with Brown OCA, which lacked a 3.3-kb transcript signal for TRP-1. *b,* Blots used for the autoradiograms in figure *5a,* which were scanned and quantitated by Image Quant™ analysis. The overloading of Caucasian RNA (i.e., control lane) was normalized by equilibrating the amount of phosphoimage intensity of the G3PDH between the samples of the Caucasian and the normal twin. Adjusted values for the Caucasian control were then set at 100% and the band intensity in the normal twin and the twin with Brown OCA lanes were quantitated accordingly. The results verify that no TRP-1 transcripts were identifiable in the twin with Brown OCA.

TRP-1 in murine melanocytes regulates the synthesis of black, insoluble melanin (Jackson 1988; Bennett et al. 1990; Orlow et al. 1992). As demonstrated in this report, the function of TRP-1 in human melanocytes may be similar: the absence of TRP-1 inhibits the synthesis of black, insoluble melanin but is permissive for formation of the "soluble" form of melanin, which is relatively more abundant in the proband. It is ironic that Coleman (1962) had originally demonstrated that skin extracts from brown mice homozygous for the *b* or the b^c alleles on either the *a/a, c/c,* or A^y/a, *C/C* background exhibited an increase in conversion of C^{14}-tyrosine to insoluble melanin over the respective *B* allele. However, Prota et al. (1995) recently demonstrated that substitution of *b* for the *B* allele dramatically reduced the amount of eumelanin in hair on either the *a/a* or *a/a* c^{2J}/c^{2J} background. As noted in the Introduction, the function of TRP-1 has been in question; it has been ascribed various catalytic functions in the mouse system, which include tyrosine hydroxylase (Jiménez et al. 1991; Jiménez-Cervantes et al. 1993; Zhao et al. 1994*b*), DOPA oxidase (Jiménez-Cervantes et al. 1993), DOPAchrome tautomerase (Winder et al. 1993), DHI oxidase (Hearing 1993), catalase (Halaban and Moellmann 1990), and, most recently, DHICA oxidase activities (Jiménez-Cervantes et al. 1994; Kobayashi et al. 1994).

Regardless of what, if any, its primary catalytic function is, TRP-1 appears to also have a regulatory role on the tyrosine hydroxylase activity of tyrosinase. As shown in this report, the tyrosine hydroxylase activity of tyrosinase is dramatically compromised in the intact cell (as demonstrated by the in situ assay) but is unaffected when the melanocyte is lysed (as demonstrated by the in vitro assay). Fixation of intact cells prior to determining tyrosine hydroxylase does not rescue its compromised tyrosine hydroxylase activity in the melanocytes from the twin with Brown OCA, which suggests that a physical interaction between TRP-1 and tyrosinase might exist within human melanocytes. This situation would be similar to that found in murine melanocytes. Hearing and colleagues (Hearing et al. 1992; Winder et al. 1994) have demonstrated that the stability

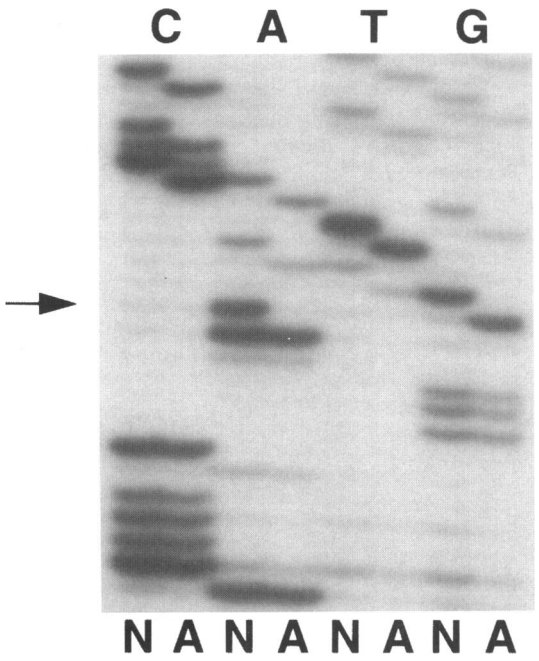

Figure 6 DNA sequencing gel of the OCA proband (A) and the unaffected normal twin brother (N). DNA from fibroblasts cultured from the proband demonstrates a mutation at codon 368 in which the A is deleted (*arrow*) resulting in a frameshift of the coding region.

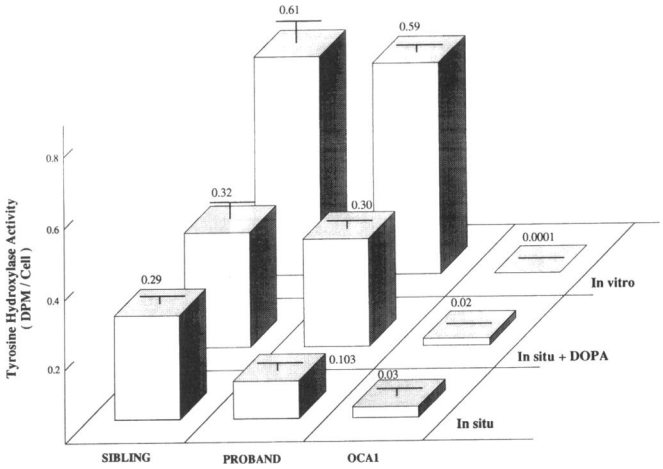

Figure 7 Tyrosine hydroxylase activities in cultured human melanocytes. Melanocyte cultures derived from the normal (SIBLING) and the brown OCA (PROBAND) twins, and a neonatal donor with tyrosinase-related albinism (OCA1) were assayed for tyrosine hydroxylase activity using melanocyte lysates (in vitro, 1 h) assay or the in situ assays with or without adding DOPA. The melanocytes from the twin with Brown OCA express ~30% the tyrosine hydroxylase activity of the normal twin when assayed by the in situ assay without the addition of DOPA but demonstrated tyrosine hydroxylase activity levels comparable to the normal twin when assayed with the addition of DOPA as well as when assayed by the in vitro assay. Cultures of melanocytes from the OCA1 individual expressed no appreciable amount of tyrosine hydroxylase with any of the assays.

of tyrosine hydroxylase activity of immunopurified tyrosinase is enhanced in the presence of TRP-1 and/or TRP-2. Orlow et al. (1993, 1994) have demonstrated that TRP-1 colocalizes with tyrosinase to melanosomes and copurifies with tyrosinase and TRP-2 as a high-molecular-weight multimer (i.e., "complex"). We were able to rescue the suppressed tyrosine hydroxylase activity of intact melanocytes cultured from the TRP-1–deficient twin with Brown OCA by providing the substrate/cofactor for tyrosinase downstream of the initial tyrosine hydroxylation step (i.e., L-DOPA) in the culture assay medium. This result suggests that TRP-1 may also be responsible for upregulating the availability of L-DOPA during the onset of melanogenesis as we have previously suggested (Zhao et al. 1994*b*, 1996). We have recently demonstrated that immunopurified TRP-1 exhibits tyrosine hydroxylase activity only with tyrosine concentrations at least 10-fold lower than that routinely used in the Pomerantz assay (Zhao et al. 1994*b*). Therefore, TRP-1 could also be catalytically involved in the efficient initiation of melanogenesis by providing the critical cofactor, L-DOPA.

In conclusion, this report demonstrates that in one individual presenting with the phenotype of Brown OCA there is a genetic, molecular, and cellular aberration in TRP-1. This is the third type of OCA to be mapped to a specific locus, thus distinguishing Brown OCA as OCA3. However, the extent of this TRP-1–associated albinism in the human population needs to be further evaluated.

Table 3

Tyrosine Hydroxylase Activities of Cultured Melanocytes

TH Activity[a]	Brown OCA Twin	Normal Twin	OCA2	OCA1
In vitro (DPM/cell/h)	3.71 ± .42	3.33 ± 1.09	3.59 ± .61	0
In situ (DPM/cell/24 h)	.33 ± .04	1.07 ± .13	.48 ± .11	.02 ± 0
Postfixation (DPM/cell/24 h)	.12 ± .01	.36 ± .03	.34 ± .07	.01 ± 0

[a] Tyrosine hydroxylase activities were performed using melanocytes derived from the Brown OCA twin, the pigmented twin, an OCA2, and an OCA1 individual as described in Patients, Material, and Methods.

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