Tyrosinase processing and intracellular trafficking is disrupted in mouse primary melanocytes carrying the *underwhite* (*uw*) mutation. A model for oculocutaneous albinism (OCA) type 4

Gertrude-E. Costin[1], Julio C. Valencia[2], Wilfred D. Vieira[1], M. Lynn Lamoreux[3] and Vincent J. Hearing[1,*]

[1]Pigment Cell Biology Section, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
[2]Pathology Section, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA
[3]Department of Veterinary Pathobiology, Texas A and M University, College Station, TX 77843, USA
*Author for correspondence (e-mail: hearingv@nih.gov)

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Summary

Oculocutaneous albinism (OCA) type 4 is a newly identified human autosomal recessive hypopigmentary disorder that disrupts pigmentation in the skin, hair and eyes. Three other forms of OCA have been previously characterized, each resulting from the aberrant processing and/or sorting of tyrosinase, the enzyme critical to pigment production in mammals. The disruption of tyrosinase trafficking occurs at the level of the endoplasmic reticulum (ER) in OCA1 and OCA3, but at the post-Golgi level in OCA2. The gene responsible for OCA4 is the human homologue of the mouse *underwhite* (*uw*) gene, which encodes the membrane-associated transporter protein (MATP). To characterize OCA4, we investigated the processing and sorting of melanogenic proteins in primary melanocytes derived from *uw/uw* mice and from wild-type mice. OCA4 melanocytes were found to be constantly secreted into the medium dark vesicles that contain tyrosinase and two other melanogenic enzymes, Tyrp1 (tyrosinase-related protein 1) and Dct (DOPAchrome tautomerase); this secretory process is not seen in wild-type melanocytes. Although tyrosinase was synthesized at comparable rates in wild-type and in uw-mutant melanocytes, tyrosinase activity in uw-mutant melanocytes was only about 20% of that found in wild-type melanocytes, and was enriched only about threefold in melanosomes compared with the ninefold enrichment in wild-type melanocytes. OCA4 melanocytes showed a marked difference from wild-type melanocytes in that tyrosinase was abnormally secreted from the cells, a process similar to that seen in OCA2 melanocytes, which results from a mutation of the *pink-eyed dilution* (*P*) gene. The P protein and MATP have 12 transmembrane regions and are predicted to function as transporters. Ultrastructural analysis shows that the vesicles secreted from OCA4 melanocytes are mostly early stage melanosomes. Taken together, our results show that in OCA4 melanocytes, tyrosinase processing and intracellular trafficking to the melanosome is disrupted and the enzyme is abnormally secreted from the cells in immature melanosomes, which disrupts the normal maturation process of those organelles. This mechanism explains the hypopigmentary phenotype of these cells and provides new insights into the involvement of transporters in the normal physiology of melanocytes.

Key words: OCA4, Underwhite, Tyrosinase, Pigmentation, Albinism

Introduction

Albinism is a heterogeneous group of genetic hypopigmentary disorders clinically divided into ocular albinism (OA) and oculocutaneous albinism (OCA). OA mainly affects pigmentation in the visual system and is considered to be a mild form of OCA (King et al., 2002). All four types of OCA discovered so far are associated with reduced/absent pigmentation of the skin, hair and eyes, which occurs as a consequence of mutations in genes involved in regulating melanin biosynthesis. Three of the OCA distinctive phenotypes known in humans have been well characterized: OCA1 (MIM #203100), OCA2 (MIM #203200) and OCA3 (MIM #203290). They have been mapped to distinct pigment genes encoding tyrosinase (Tyr), the pink-eyed dilution (P) protein and tyrosinase-related protein 1 (Tyrp1), respectively. OCA4 (MIM #606574) is a very recently discovered form of OCA and is associated with mutations in the *underwhite* (*uw*) gene, which encodes the membrane-associated transporter protein (MATP) (Newton et al., 2001). MATP is regulated at the transcriptional level by Mitf, a basic helix-loop-helix transcription factor that modulates the expression of a number of melanocyte-specific genes, such as *Tyr* and *Tyrp1* (Baxter and Pavan, 2002). The uw mutation has been shown to significantly reduce the melanin content of skin and hair in humans and in mice (Lehman et al., 2000; Newton et al., 2001). MATP has 12 predicted membrane-spanning regions, as does the P protein associated with OCA2.

OCA1 is an autosomal recessive disorder associated with

deficient catalytic function of tyrosinase, the rate-limiting enzyme in melanin biosynthesis, which results from mutations of its encoding gene (*TYR*). Individuals with OCA1 show a complete absence of pigment in their skin, eyes and hair; this persists throughout life in the subtype OCA1A (Spritz et al., 1990), whereas patients with subtype OCA1B have some residual tyrosinase activity and thus gradually accumulate minor amounts of pigment in their tissues (Oetting and King, 1999). OCA2 is the most common form of albinism; patients are usually born with minimal pigmentation and become darker during adulthood, finally showing minimal-to-moderate pigmentation of their skin, hair and eyes (King, 1998; Manga and Orlow, 1999). OCA3 is a rare form of OCA and patients are born with minimal pigmentation but increase their pigmentation as they age (Boissy et al., 1996; Manga et al., 1997).

Studies using immortalized melanocytes derived from mice carrying mutations in the genes responsible for OCA1 and OCA3 have shown that these disorders are endoplasmic reticulum (ER) retention diseases (Halaban et al., 2000; Toyofuku et al., 2001a; Toyofuku et al., 2001b), wherein tyrosinase is abnormally targeted for digestion in proteasomes rather than being transferred to the Golgi apparatus for further glycosylation. By contrast, studies of immortalized p-mutant melanocytes (the model for OCA2) have shown that tyrosinase in those cells is correctly processed through the Golgi, but it is then secreted from the cells rather than being sorted to melanosomes, thus showing that OCA2 is a hypopigmentary disease resulting from altered intracellular trafficking of tyrosinase (Manga et al., 2001; Chen et al., 2002; Toyofuku et al., 2002). In this context, investigations into the molecular basis of OCA4 are necessary to classify its mechanism into one of the above types of diseases or into yet a new distinct type.

In this study, we established primary melanocyte cultures from mice carrying the *uw/uw* mutation at the *underwhite* locus and from wild-type mice as a control. We show that in uw-mutant melanocytes, tyrosinase processing through the ER and Golgi is normal but that subsequent intracellular trafficking to melanosomes is aberrant and a significant amount of tyrosinase (and other melanogenic enzymes) is secreted from the cells in vesicles and immature melanosomes, rather than melanin being produced intracellularly in mature melanosomes. Thus, the molecular basis of OCA4 shows a mechanism similar to that involved in OCA2 but which occurs later in the melanosomal maturation pathway, and in this context our results provide new and important insights about the involvement of transporters in the normal physiology of melanocytes.

Materials and Methods

Cell culture

Primary melanocytes from *a/a; Uw/Uw; Tyr/Tyr* (wild-type), *a/a; uw/uw; Tyr/Tyr* (uw-mutant) and *a/a; Uw/Uw; tyr^c/tyr^c* (albino) C57BL/6J mice were derived from dorsal skins of 1 day old mice using a method previously reported (Tamura et al., 1987). Briefly, dissected skins were washed twice with 70% ethanol, twice with phosphate buffer saline (PBS) and were then individually incubated in 6 ml of 0.25% trypsin at 4°C overnight. The epidermis was then removed and the dermis was cut into small pieces and placed in Ham's F-10 medium (GIBCO, Grand Island, NY) for 2 days, at 37°C in a humidified incubator with 5% CO_2. The Ham's F-10 medium contained 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 10% newborn calf serum (Invitrogen, Carlsbad, CA), 0.1 mM isobutylmethyl xanthine (Sigma, St Louis, MO), 10 μg/ml bovine pituitary extract (Invitrogen), 48 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (Sigma), 1% penicillin/streptomycin/fungizone (Sigma) and 1 mM glutamine (Invitrogen). After 2 days, tissue debris was removed by suction and the medium was changed and supplemented with 100 μg/ml geneticin G418 (G418 sulphate, Invitrogen). The geneticin treatment lasted 2-3 days and was repeated intermittently if any fibroblasts or keratinocytes persisted. Once pure colonies of melanocytes were obtained, the medium was changed to 'maintenance' medium (Ham's F-10) which contained 20% fetal bovine serum, 0.1 mM dibutyryladenosine 3′,5′-cyclic monophosphate (Sigma), 10 μg/ml bovine pituitary extract, 48 nM 12-*O*-tetradecanoyl-phorbol-13-acetate, 1% penicillin/streptomycin/fungizone and 1 mM glutamine. Cells were cultured in this medium during the experiments.

Antibodies and enzymes

αPEP1, αPEP7 and αPEP8 are rabbit antisera raised in our laboratory against the carboxyl-terminal peptides of Tyrp1, Tyr and Dct (DOPAchrome tautomerase), respectively, as previously described (Jiménez et al., 1989; Tsukamoto et al., 1992). Anti-rabbit IgG horseradish peroxidase-linked (whole antibody) was from Amersham Pharmacia Biotech (Piscataway, NJ), KDEL (SPA-827) polyclonal antibody was purchased from StressGen (Victoria, BC, Canada) and HMB-45 monoclonal antibody (Schaumburg-Lever et al., 1991; Kikuchi et al., 1996) was purchased from DAKO (Carpinteria, CA). Normal horse serum, normal goat serum, Texas-red anti-rabbit IgG (H+L) and fluorescein isothiocyanate anti-mouse IgG (H+L) were all from Vector (Burlingame, CA). Endoglycosidase H (EndoH) [EC 3.2.1.96, glycopeptide-D-mannosyl-N^4-(*N*-acetyl-D-glucosaminyl)$_2$-asparagine 1,4-*N*-acetyl-β-glucosaminohydrolase] and peptide:*N*-glycosidase F (PNGaseF) [EC 3.5.15.2, *N*-linked-glycopeptide-(*N*-acetyl-β-D-glucosaminyl)-L-asparagine amidohydrolase] were from New England Biolabs (Beverly, MA).

Cellular fractionation

Melanocytes were harvested with trypsin/EDTA and were washed three times with cold PBS without $CaCl_2$ and $MgCl_2$. Cells were solubilized in extraction buffer [1% Nonidet P-40 in PBS containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany)] for 1 hour on ice with occasional vortexing. The samples were centrifuged at 14,000 ***g*** for 30 minutes at 4°C and the supernatants were recovered and kept at –70°C for further experiments. Protein concentrations were determined with the BCA assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

To collect vesicles secreted by melanocytes, medium collected from cells cultured for one week was centrifuged at 14,000 ***g*** for 30 minutes at 4°C. The precipitates were solubilized in the extraction buffer and were stored as described above.

To isolate the melanosome-enriched fraction, we used a method previously described (Valverde et al., 1992). Briefly, the cells were harvested, washed three times with PBS without $CaCl_2$ and $MgCl_2$ and lysed with 10 mM phosphate buffer, pH 7.2, containing 0.25 M sucrose. A postnuclear supernatant was obtained by centrifugation at 700 ***g*** for 15 minutes at 4°C and was then further fractionated by centrifugation at 14,000 ***g*** for 30 minutes at 4°C to yield the melanosome-enriched pellet.

Metabolic labeling

Metabolic labeling and immunoprecipitation experiments were

performed as previously reported (Kobayashi et al., 1994). Briefly, melanocytes were cultured in 6-well tissue culture plates for 48 hours before labeling. The cells were then incubated in methionine- and cysteine-free Dulbecco's Modified Eagle Medium (GIBCO) for 30 minutes at 37°C in a humidified incubator with 5% CO_2, and were then labeled for 1 hour with 0.5 mCi of [^{35}S]-Methionine and Cysteine Mixture (Redivue Promix, Amersham Biosciences). Following the 1 hour labeling period, the isotope-supplemented medium was removed and complete medium containing 1 mM unlabeled methionine was added. Media were then collected and the cells were harvested by scraping immediately and after 3 or 24 hours of chase. The cells were washed three times with PBS at 4°C and then solubilized for 1 hour at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 1% Nonidet P-40, 0,01% SDS and protease inhibitor cocktail). [^{35}S]-labeled cell lysates were precleared with 20 μl Protein-G Sepharose 4 Fast Flow (Amersham) for 2 hours at 4°C with mixing. The supernatants were collected following centrifugation at 5000 ***g*** for 1 minute at 4ºC and then incubated with αPEP1 or *α*PEP7 for 2 hours at 4°C with mixing, as were the media collected at each of the chase periods. The immune complexes were separated by incubation with 20 μl Protein-G Sepharose 4 Fast Flow for 2 hours at 4°C and were further washed three times by centrifugation with the lysis buffer. The final pellets were resuspended in sample buffer, heated at 100°C for 5 minutes and centrifuged. The samples were separated on 8% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide electrophoresis) gels. The dried gels were exposed in a storage phosphor screen, scanned with a STORM Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) and then analyzed with the Image Quant program.

Western blotting

For western blotting, samples were separated by electrophoresis under reducing conditions, as previously described (Negroiu et al., 1999). Briefly, lysates were mixed 1:2 (v/v) with sample buffer (BioRad, Hercules, CA) containing 2-mercaptoethanol and were boiled for 10 minutes. Samples were separated by PAGE in SDS (8% Tris-glycine gels, Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked in 5% non-fat milk in PBS for 1 hour at room temperature and were then incubated with the appropriate first antibodies diluted 1:1000 in 1% non-fat milk in PBS, for 1 hour at room temperature. After six washes (10 minutes each) with 0.05% Tween 20 (Bio-Rad) in PBS, the blots were incubated in anti-rabbit horseradish peroxidase-linked whole antibody (1:1500) (Amersham) in 1% non-fat milk in PBS for 1 hour at room temperature. After three further washes (10 minutes each) with 0.05% Tween 20 in PBS, the immunoreactivity of the blots was detected using an Enhanced ChemiLuminescence western blotting detection kit (Amersham), according to the manufacturer's instructions.

EndoH and PNGaseF digestions

Samples to be digested were denatured in the EndoH- or PNGaseF-denaturing buffer for 5 minutes at 100°C, cooled and then mixed with 1/10 concentrated EndoH (0.5 M sodium citrate, pH 5.5) or PNGaseF [0.5 M sodium phosphate, pH 7.5 containing 10% (v/v) Nonidet P40] reaction buffers as previously described (Negroiu et al., 1999). Samples were then digested with 500 units of EndoH or PNGaseF, respectively, for 3 hours at 37°C and analyzed by PAGE.

Melanogenic assays

Tyrosinase enzymatic activity was measured as described previously (Hearing and Ekel, 1976). Briefly, 30 μl of each sample to be tested were added to 10 μl phosphate buffer containing *β*-3,4-dihydroxyphenylalanine (DOPA) as cofactor and 10 μl 0.25 mCi/mmol [U-^{14}C]-tyrosine (NEN, Boston, MA). The reactions (usually performed in duplicate or triplicate) were mixed and incubated at 37°C for 3 hours. 40 μl of each sample were transferred to Whatman 3MM filter paper discs and air dried. Each filter was then washed three times in 0.1 N HCl, twice in 95% ethanol, once in acetone and finally air-dried. Radioactive melanin retained by the filters was determined in a Beckmann scintillation counter. Tyrosinase activity is defined as pmol [^{14}C]-tyrosine incorporated into melanin/μg protein/hour at 37°C.

The melanin content was measured using a modification of a previously reported method (Siegrist and Eberle, 1986). Briefly, melanocytes were cultured until they became confluent. They were solubilized in lysis buffer containing the protease inhibitor cocktail and protein concentrations were measured. Melanin pellets were dissolved by incubation in 1 N NaOH at 37°C for 18 hours. Aliquots of each sample were transferred to 96-well plates and were quantitated by absorbance at 405 nm using an automatic microplate reader (Molecular Devices, Sunnyvale, CA) and calibrated against a standard curve generated using synthetic melanin (Sigma).

Immunohistochemical staining

Dual labeling, using immunofluorescence methods and laser scanning confocal fluorescence microscopy, was used to evaluate the localization of melanogenic proteins in wild-type and in *uw/uw* melanocytes, as previously reported (Kushimoto et al., 2001). The following antibody dilutions were used: *α*PEP1, 1:40; *α*PEP7, 1:40; *α*PEP8, 1:40; KDEL, 1:10; HMB-45, 1:10. Melanocytes were plated in 2-well Lab-Tek chamber glass slides (Nalge, Naperville, IL) 24-72 hours before each experiment and were fixed in 4% paraformaldehyde for 15 minutes at 4°C. Cells were subsequently permeabilized with methanol for 20 minutes at 4°C and then with 1% Triton X-100 for 3 minutes at room temperature (in experiments using the KDEL antibody); all subsequent processing was done at room temperature. After washing in PBS, cells were incubated in PBS containing 5% normal serum (normal horse serum for monoclonal antibodies and normal goat serum for polyclonal antibodies) for 1 hour as a blocking step. The cells were then incubated overnight at 4°C with the appropriate first antibody diluted in 2% normal serum. This step was followed by incubation with the appropriate secondary antibody (dilution, 1:100) for 1 hour. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Vector – blue fluorescence) for 15 minutes at room temperature. Immunoreactive cells were classified into three categories according to whether they showed green, red or yellow fluorescence (the latter color indicating colocalization of the red and green signals). All preparations were examined with a Model TCS4D, DMIRBE confocal microscope (Leica, Heidelberg, Germany) equipped with argon and argon-krypton laser sources. Controls included sections stained as detailed above, but omitting the first antibody.

Electron microscopy

Confluent flasks of wild-type and of uw-mutant melanocytes were fixed with 2% glutaraldehyde for 24 hours at 4°C, collected by centrifugation and then washed twice with cold PBS. Further, analysis of uw-mutant melanocytes was performed using the DOPA reaction, an enzyme histochemical method to detect tyrosinase activity. For DOPA staining, melanocytes were incubated for 3 hours at 37°C in 0.1% L-DOPA in 0.1 M phosphate buffer, pH 7.4, and fixed in 2% glutaraldehyde as above. All samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for 1 hour, dehydrated through a graded ethanol series and embedded in PolyBed 812 at 60°C for 48 hours. Ultrathin sections were cut and stained with uranyl acetate and were then examined in a JEOL JEM-1200 EX electron microscope.

Results

Morphology of wild-type and uw-mutant primary melanocytes in culture

Primary melanocytes derived from skins of wild-type and from *uw/uw* mice were cultured under identical conditions, and cellular viability and morphological characteristics were noted. The growth of both types of melanocytes were quite similar, with doubling times of approximately 10 days, and their morphologies were quite stable over continued passage (Fig. 1). Both types of melanocytes grew as an adherent monolayer and were dendritic, although the uw-mutant melanocytes were significantly larger and more dendritic compared with wild-type melanocytes (cf. below). Bright-field microscopy showed a very pigmented population of wild-type melanocytes, whereas the uw-mutant cells were only very lightly pigmented. Dark vesicles could be detected that were constantly secreted into the medium by uw-mutant melanocytes, whereas none were evident in the medium of wild-type melanocytes.

The pattern of melanin present in the extracellular medium was similar to but distinct from that previously shown for OCA2 p-mutant melanocytes (Potterf et al., 1998). In OCA2 p-mutant melanocytes, active tyrosinase was secreted into the medium and the melanin produced in the medium was of very low molecular weight and could not be sedimented by centrifugation. By contrast, the pigment present in the medium of uw-mutant melanocytes was in large particles (Fig. 1F) that could be readily sedimented by centrifugation (Fig. 1H). We next characterized the nature of tyrosinase synthesis and processing to elucidate the intracellular mechanism(s) responsible for the hypopigmentation of uw-mutant melanocytes.

Subcellular distribution of tyrosinase activity and melanin in wild-type and uw-mutant melanocytes

Wild-type and uw-mutant melanocytes were cultured until they became confluent, and they were then fractionated and solubilized in lysis buffer. Tyrosinase activity was measured in the cell extracts and also in the melanosome and secreted vesicle fractions (Table 1). In wild-type melanocytes, the specific activity of tyrosinase in melanosomes was approximately ninefold higher than in the cell extract, which is the normal distribution pattern for tyrosinase trafficking. Tyrosinase activity in the vesicle fraction secreted by wild-type melanocytes was very low and represented less than 1% of the

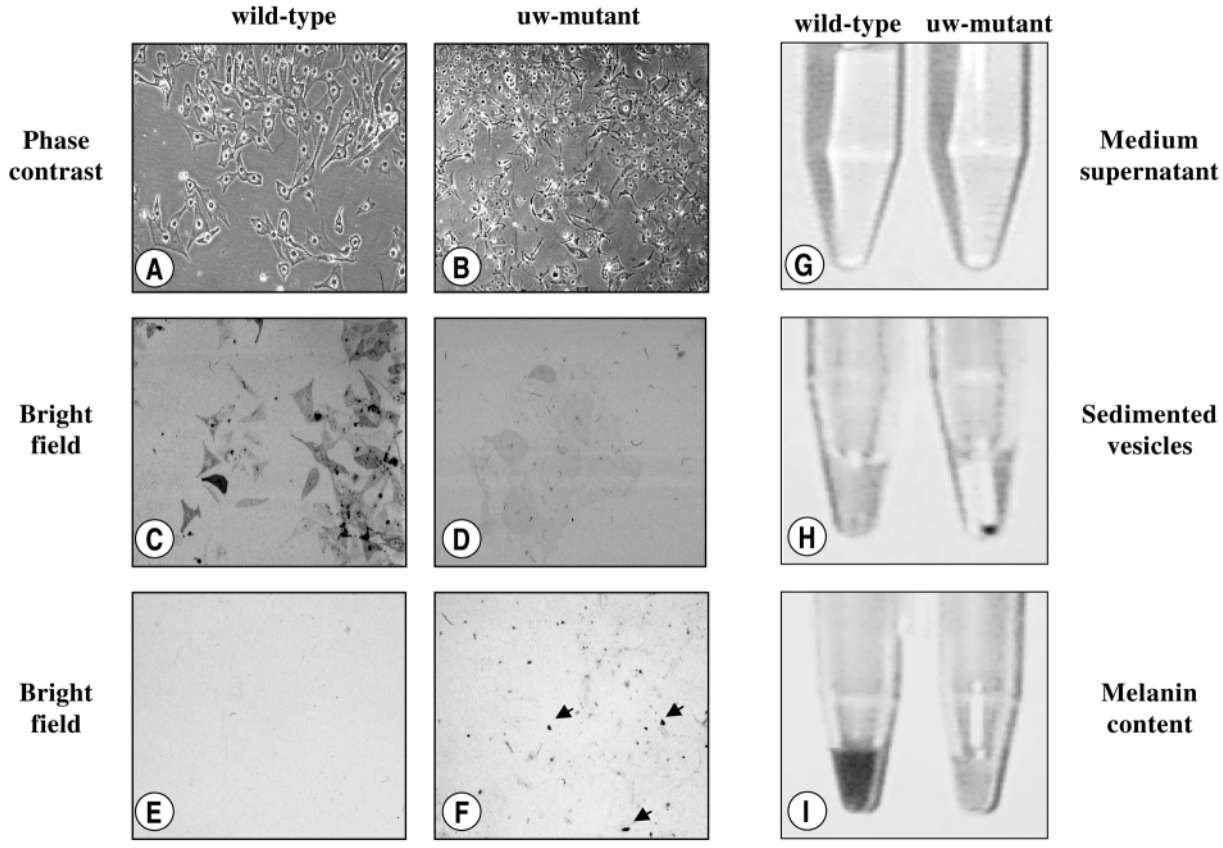

Fig. 1. Characteristics of wild-type and uw-mutant primary murine melanocytes in culture. (A,B) Phase contrast photomicrographs of wild-type and uw-mutant melanocytes. (C,D) Bright-field microphotographs emphasizing the pigmentation of wild-type and uw-mutant melanocytes. (E,F) Bright-field microphotographs showing the pigmented vesicles secreted by uw-mutant melanocytes into the medium (arrows) and their absence in the medium of wild-type melanocytes. All photomicrographs are shown at the same magnification. (G) Media were collected from wild-type and from uw-mutant melanocytes, and were centrifuged to show the similar appearance of those samples (i.e. no soluble melanin was present). (H) Pigmented vesicles released into the medium by uw-mutant melanocytes can be readily sedimented by centrifugation, a process not seen in wild-type melanocytes. (I) Melanin content of extracts of wild-type and uw-mutant melanocytes.

Table 1. Tyrosinase activity and melanin content in subcellular fractions of wild-type vs uw-mutant melanocytes

	Wild-type melanocytes		Uw-mutant melanocytes	
Fraction	Tyrosinase activity (pmol/μg protein/h)	Protein concentration (μg/μl)	Tyrosinase activity (pmol/μg protein/h)	Protein concentration (μg/μl)
Cell extract	66.61±0.50	0.53±0.00	14.27±2.26	0.94±0.01
Melanosomes	590.99±0.01	0.37±0.00	46.92±0.45	0.14±0.00
Secreted vesicles	0.51±0.16	0.27±0.01	0.88±0.66	0.11±0.00
	Melanin content (μg/μg protein)	Protein concentration (μg/μl)	Melanin content (μg/μg protein)	Protein concentration (μg/μl)
	2.79±0.06	1.30±0.01	0.01±0.00	2.74±0.11

activity seen in the total extract. By contrast, tyrosinase activity in extracts of uw-mutant melanocytes was significantly lower (about fivefold lower) than that found in wild-type melanocytes. Tyrosinase activity in melanosomes of uw-mutant melanocytes was only threefold higher than that found in the total cell extract, and more than 5% of the enzyme activity was present in the secreted vesicle fraction. The total melanin content in uw-mutant melanocytes is negligible compared with wild-type melanocytes (Table 1), which is consistent with the report of Lehman et al. (Lehman et al., 2000).

Synthesis and processing of melanogenic proteins in uw-mutant and wild-type melanocytes

Thus, the melanin content of uw-mutant OCA4 melanocytes is consistent with their hypopigmented phenotype and also with the reduced levels of tyrosinase and melanin found in OCA1, OCA2 and OCA3 melanocytes. To further study the mechanism behind that reduction, we used metabolic labeling and western immunoblotting to examine the synthesis and processing of tyrosinase in uw-mutant compared with wild-type melanocytes. As shown in Fig. 2, the amount of tyrosinase synthesized (at 0 chase time) by wild-type and by uw-mutant melanocytes was virtually identical, showing that there was no defect in tyrosinase gene transcription or translation, as expected. Quantitation of the 64 kDa band representing the newly synthesized tyrosinase revealed that the amount of tyrosinase synthesized by uw-mutant melanocytes represents ~90% the level produced by wild-type melanocytes (n=4 independent experiments). Tyrosinase is synthesized as a 55 kDa polypeptide, which almost instantly undergoes early glycosylation events in the ER and is seen as a 64 kDa band. The fully glycosylated mature form of the enzyme is 68 kDa in size. In fact, the synthesis and processing of tyrosinase from the immature form to the mature form was complete within 3 hours in wild-type and in uw-mutant melanocytes and was strikingly similar at that time. However, the stability of tyrosinase was markedly different, with virtually 100% of the tyrosinase remaining in wild-type melanocytes after 24 hours, but very little remaining in uw-mutant cells. For comparison, the pattern for OCA1 melanocytes is shown, wherein tyrosinase was synthesized at the same rate as in wild-type and in uw-mutant melanocytes. However, tyrosinase was not processed to the mature form correctly and was then quickly degraded, as previously reported (Toyofuku et al., 2001b).

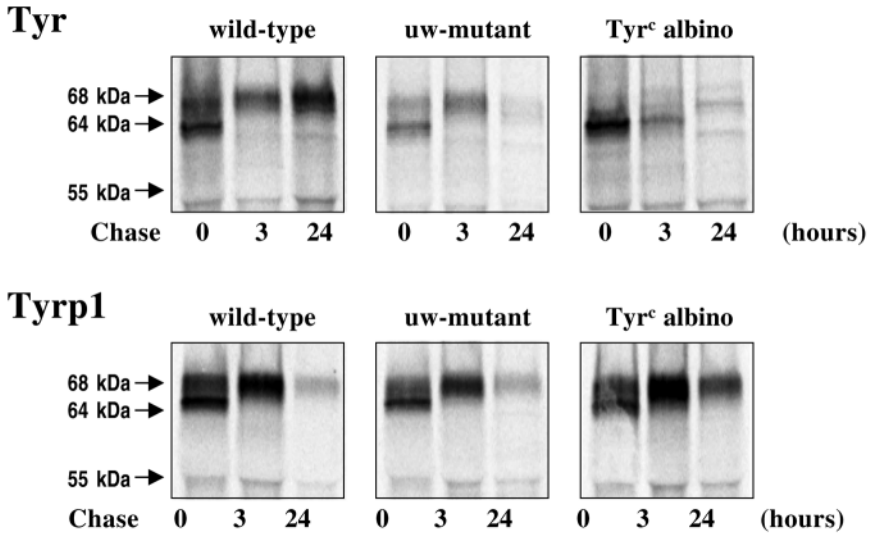

Fig. 2. Metabolic pulse-chase labeling of wild-type and uw-mutant melanocytes. Wild-type, uw-mutant and Tyr^c albino primary melanocytes were labeled for 1 hour with an [^{35}S]-methionine and cysteine mixture, harvested at the chase times indicated, solubilized and immunoprecipitated using specific antibodies for tyrosinase and Tyrp1, respectively. Immune complexes were separated by SDS-PAGE, and immunoprecipitated bands were visualized as detailed in the Materials and Methods section.

Similar patterns of Tyrp1 synthesis and processing are shown for comparison, and no significant differences in Tyrp1 labeling were seen between the wild-type and OCA4 (or OCA1) melanocytes. The synthesis, processing and stability of Dct was also virtually identical in all three types of melanocytes (data not shown).

Western blot analysis revealed that tyrosinase was detectable predominantly in the mature 68 kDa form in the whole cell extract and only in the mature form in melanosomes of wild-type melanocytes, and none was detected in the secreted vesicle fraction (Fig. 3). By contrast, tyrosinase was detectable not only in the 68 kDa form in the cell extract and in melanosomes of uw-mutant melanocytes but also in the secreted vesicle fraction. Interestingly, Tyrp1, although processed correctly and found in melanosomes of uw-mutant melanocytes, was also present in abundance in the secreted vesicle fraction of those uw-mutant

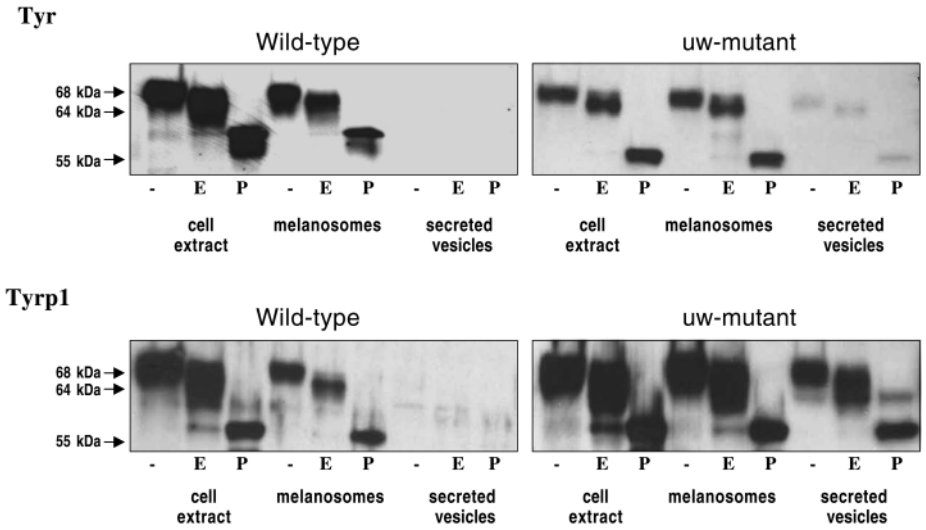

Fig. 3. Western blot analysis of melanogenic protein glycosylation in subcellular fractions of wild-type and of uw-mutant melanocytes. Equal amounts of protein (0.1 μg/lane) from cell extracts, melanosomes and secreted vesicles of wild-type or uw-mutant melanocytes were electrophoretically separated by 8% SDS-PAGE. Tyrosinase or Tyrp1 were then detected immunochemically with αPEP7 or αPEP1 antibodies, respectively. Glycosylation of melanosomal proteins was determined following digestion with buffer (–), EndoH (E) or PNGaseF (P), as indicated.

cells but was not found in the secreted vesicle fraction of wild-type melanocytes. Similar patterns of Dct distribution were seen in those vesicles (not shown).

Subcellular trafficking of tyrosinase in OCA4 melanocytes

To examine more closely the mechanism(s) by which tyrosinase is aberrantly secreted from the uw-mutant melanocytes, we assessed the sensitivity of tyrosinase to two different glycosidases. EndoH is an enzyme that removes high mannose-type carbohydrates from *N*-linked glycoproteins, a conversion that occurs in the medial Golgi region. When proteins are correctly processed through the ER and Golgi, they become resistant to EndoH, and sensitivity to EndoH indicates the presence of proteins that have not yet been processed beyond the ER. By contrast, PNGaseF removes all carbohydrate residues and reveals the native polypeptide size of the protein. When digested with EndoH (Fig. 3), the mature 68 kDa form of tyrosinase from the cell extract of uw-mutant melanocytes was almost completely resistant to EndoH but was completely digested with PNGaseF, showing a pattern virtually identical to that of wild-type melanocytes.

Western blot analysis of tyrosinase in melanosomes of wild-type and uw-mutant melanocytes revealed that the mature 68 kDa band was EndoH resistant, as expected, whereas digestion with PNGaseF generated a single strong 55 kDa band representing the fully deglycosylated form. Similarly, tyrosinase in the secreted vesicle fraction was resistant to EndoH but could be fully digested to the 55 kDa nascent polypeptide by PNGaseF. Thus, tyrosinase found in melanosomes and in vesicles secreted from uw-mutant melanocytes is EndoH resistant, showing that it has been processed correctly through the ER. Similar digestions of Tyrp1 (Fig. 3) and Dct (data not shown) showed comparable processing patterns.

Taken together, the sum of these results suggests that tyrosinase (and Tyrp1 and Dct) are processed correctly through the ER and Golgi in uw-mutant melanocytes but that a significant portion of those melanogenic enzymes are secreted from uw-mutant melanocytes. Thus, the subcellular distribution and enzyme functions of melanosomal proteins are distinctly affected by this mutation in the *uw* gene.

Subcellular distribution of melanogenic proteins in uw-mutant melanocytes

To investigate the subcellular localization of melanogenic proteins in uw-mutant OCA4 melanocytes, we used immunohistochemical staining to compare their distribution with HMB-45, a marker for early (stage II) melanosomes (Schaumburg-Lever et al., 1991; Kushimoto et al., 2001) and KDEL, a marker for the ER (Fig. 4). Tyrosinase, Tyrp1 and Dct were detected by red fluorescence, and HMB-45 and KDEL were detected by green fluorescence; in the merged images, yellow indicates colocalization of the two signals.

In wild-type primary melanocytes, the majority of tyrosinase was found in granules distributed throughout the cytoplasm (mainly in the dendrites), as well as in the perinuclear area where the ER and stage I melanosomes are found. Some tyrosinase colocalized with the ER marker KDEL but most of it was in the particulate structures. Similar staining patterns were observed for Tyrp1 and for Dct in those wild-type melanocytes. These data confirm that the melanogenic proteins are processed in the ER and are then distributed primarily in melanosomes of wild-type primary melanocytes according to their expression level, as previously shown for immortalized melanocytes (Toyofuku et al., 2001b; Toyofuku et al., 2002).

Confocal microscopy of uw-mutant melanocytes showed dramatic differences compared with wild-type melanocytes. As noted above, these cells were more dendritic and grew to much larger sizes than wild-type melanocytes, and this was particularly evident when they were cultured on glass. All three melanogenic proteins (Tyr, Tyrp1 and Dct) were distributed throughout the cytoplasm of uw-mutant melanocytes and accumulated in the perinuclear area, as in wild-type melanocytes. Colocalization of tyrosinase, Tyrp1 and Dct with the ER marker KDEL indicates that the melanogenic proteins reach the ER compartment in uw-mutant melanocytes. However, in contrast to wild-type melanocytes, there was little co-staining of tyrosinase with HMB-45, suggesting a trafficking defect later in the secretory pathway, before delivery

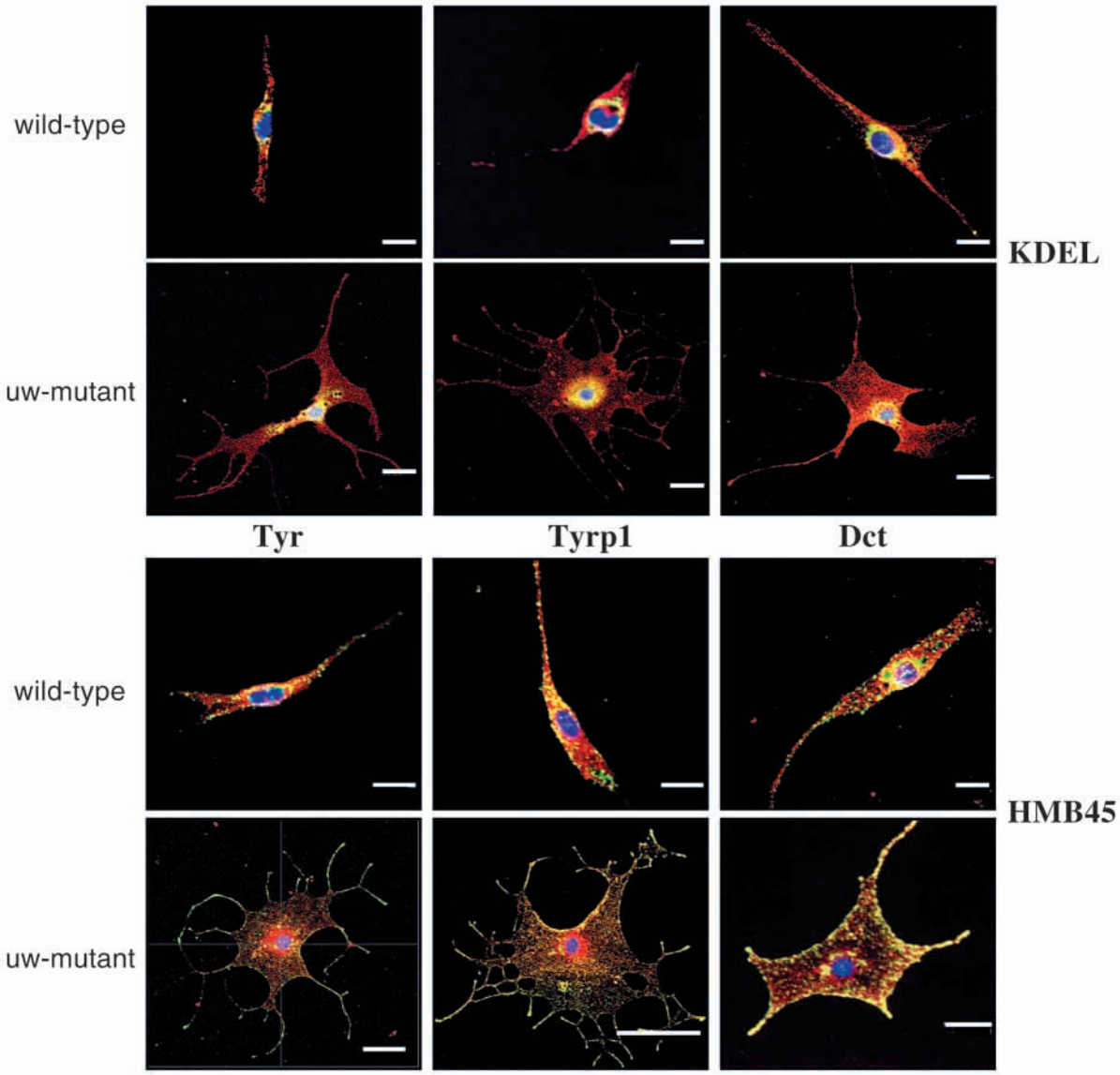

Fig. 4. Subcellular distribution of melanogenic proteins in wild-type and uw-mutant primary melanocytes. Wild-type and uw-mutant melanocytes were stained with antibodies and fluorescent probes as noted in the Materials and Methods section, and localization of tyrosinase, Tyrp1 or Dct (red) with KDEL or HMB-45 (green) was analyzed by confocal microscopy. Colocalization of antibodies is indicated by the yellow color. Nuclei are identified by the blue DAPI stain. Bar, 20 μm.

of tyrosinase to the melanosomal compartment. Tyrp1 (and to a lesser extent Dct) was similarly disrupted from reaching the melanosomal compartment, and this was especially apparent at the periphery of the cells and in the dendrites, where they did not reach early stage II melanosomes (stained green with HMB-45).

Ultrastructural characterization of melanocytes and melanosomes

Electron microscopy revealed that melanocytes derived from wild-type mice contain numerous highly melanized melanosomes (stages III and IV) with a very well-organized structure (Fig. 5A). By contrast, uw-mutant melanocytes contain swollen, poorly melanized melanosomes which show an abnormal arrangement of their internal matrices, with the majority of them being at stages I or II (Fig. 5B). DOPA reactivity of uw-mutant melanocytes showed DOPA-positive reactions in some of the stage I or II melanosomes, which is evidence that at least some active tyrosinase was correctly delivered there (Fig. 5C). Ultrastructural analysis of the vesicles shed by uw-mutant cells revealed the presence of stage I and II melanosomes exhibiting small amounts of melanin and disorganized structures of their internal fibers (Fig. 5D).

Discussion

One important aspect of this study is the use of primary melanocytes rather than immortalized cell lines to examine the processing and subcellular localization of melanogenic

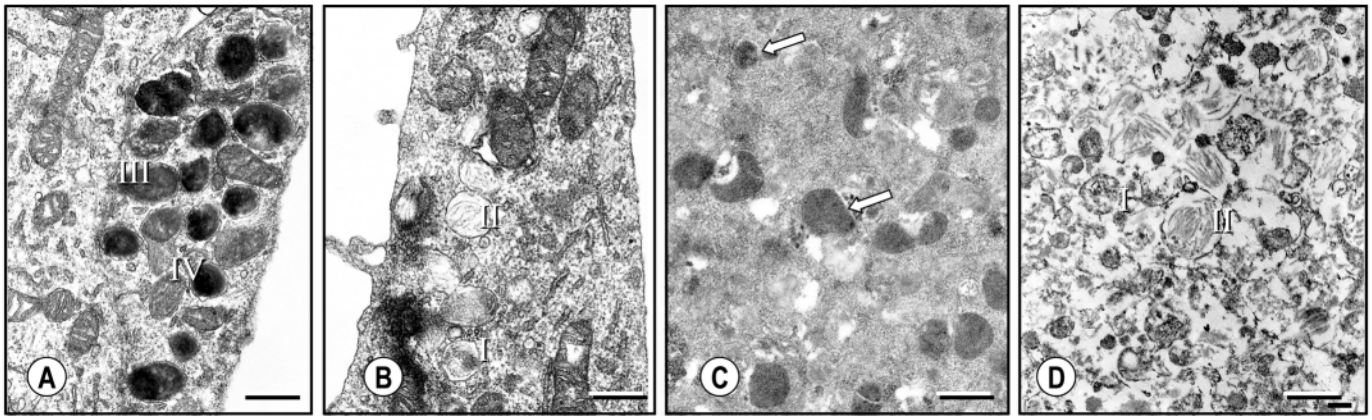

Fig. 5. Ultrastructure of primary melanocytes established from wild-type and from uw-mutant mice. (A) Wild-type melanocytes showing numerous stage III and IV melanosomes. (B) uw-mutant melanocytes displaying primarily stage I and II melanosomes. (C) uw-mutant melanocytes incubated with DOPA show a dark staining in stage I and II melanosomes (arrows), which proves the presence of active tyrosinase. (D) Secreted vesicle fraction from uw-mutant melanocytes where stage I and II melanosomes, similar to those found in (B), are identified clearly. Bar, 20 μm.

proteins. We used primary melanocytes derived from wild-type and from Tyr^{c} albino mice (as controls) and from uw-mutant mice because no immortal melanocyte line is so far available for uw-mutant OCA4 cells. Importantly, previous studies of melanosomal protein processing and sorting have been performed on immortalized or transformed melanocytes, and this study has now confirmed those results for Tyr, Tyrp1 and Dct in normal primary melanocytes. Our study represents one of the first steps in characterizing the mechanism of OCA4 and provides important clues into the pathogenesis of that hypopigmentary disorder. Eventually, we hope that these OCA4 uw-mutant melanocytes will become immortalized, which would make further studies on this type of OCA much easier. OCA4 is a newly identified type of oculocutaneous albinism which is associated with mutations of the *underwhite* (*MATP*) gene and which greatly reduce or abolish tyrosinase catalytic activity and melanin production (Lehman et al., 2000). In this study, we analyzed the intracellular processing and trafficking of melanogenic proteins in primary uw-mutant melanocytes as a model for OCA type 4, focusing on tyrosinase, the key rate-limiting enzyme in the melanin biosynthetic pathway. Melanin synthesis in mammalian melanocytes is the result of a complex metabolic pathway in which at least three enzymes, known as tyrosinase-related proteins (TRPs), are involved (Hearing and Tsukamoto, 1991; Spritz and Hearing, 1994). Tyrosinase, Tyrp1 (originally TRP1) and Dct (originally TRP2) are synthesized on ribosomes and are transported through the ER and Golgi apparatus where their post-translational processing and glycosylation take place, as schematically presented in Fig. 5. They are then sorted from the trans-Golgi network to early melanosomes by secretory vesicles and are then localized in the limiting membrane of these organelles. After the delivery of the melanogenic enzymes to melanosomes, the synthesis of melanin usually begins.

The biogenesis of melanosomes has gradually been elucidated, and the earliest form of that organelle is the stage I melanosome, which is positive for staining of gp100 with αPEP13 (Kushimoto et al., 2001). The TRPs are not delivered to stage I melanosomes, but rather arrive there only after the maturation of stage I to stage II melanosomes, which is linked to the proteolytic cleavage of gp100 (which generates the

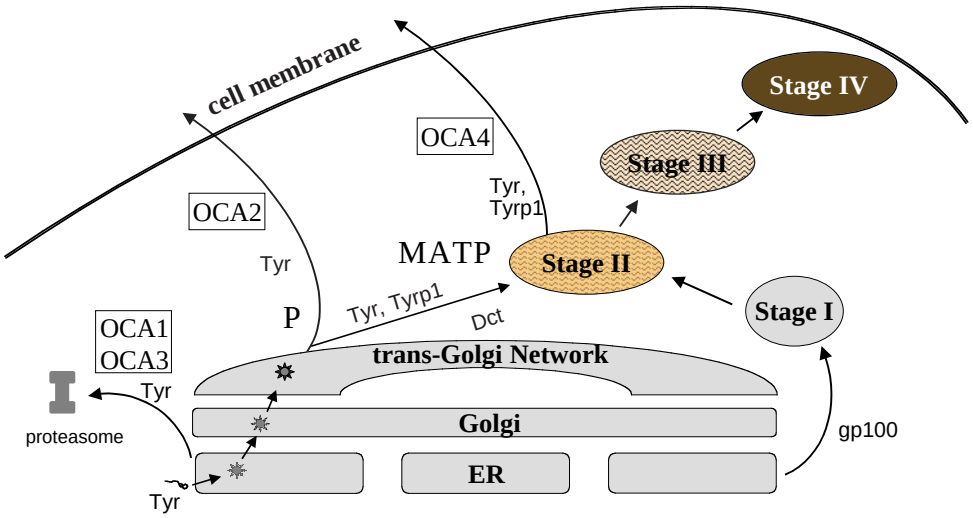

Fig. 6. Melanosomal protein trafficking and aberrant processing seen in various types of OCA. The disruption of tyrosinase trafficking occurs at the level of the ER in OCA1 and OCA3, and at the immediate post-Golgi level in OCA2. The results of this study show that in OCA4, tyrosinase processing and trafficking is disrupted and that the enzyme is abnormally secreted from the cells within vesicles before delivery to early melanosomes.

internal fibrillar matrix). Following that cleavage, gp100 undergoes a conformational change that allows it to be recognized by the monoclonal antibody HMB45, which thus serves as a specific probe for identifying stage II melanosomes (Kushimoto et al., 2001).

According to our results, uw-mutant cells express normal levels of tyrosinase, which is, in part, delivered to and is active in melanosomes. At least part of the active, correctly processed tyrosinase remains inside the melanocytes and contributes to the low levels of melanin produced, thus explaining the reduced but significant pigmentation seen in uw-mutant melanocytes. However, uw-mutant melanocytes secrete into the medium dark vesicles that contain tyrosinase, Tyrp1 and Dct. Although metabolic labeling revealed that the amount of tyrosinase synthesized in wild-type and uw-mutant melanocytes is approximately the same, tyrosinase activity in uw-mutant melanocytes is reduced to about 20% of the level found in wild-type melanocytes due to its misrouting along the secretory pathway. One can see using confocal immunohistochemistry that while some of the tyrosinase in uw-mutant cells reaches stage II (HMB45-positive) melanosomes, most of it does not, thus leading to the conclusion that the uw-encoded protein (MATP) functions in the sorting of tyrosinase (and Tyrp1) from the trans-Golgi network to stage II melanosomes. Two relevant observations should be noted about MATP in this regard. First, MATP obviously functions to sort only melanocyte-specific proteins because if other common proteins were similarly affected, the phenotype would be that of Hermansky-Pudlak syndrome rather than of OCA (Sarangarajan et al., 2001; Huizing et al., 2002). Second, a proteomic analysis of stage II melanosomes recently published by our group (Basrur et al., 2003) identified all the early melanosome-specific proteins, but MATP (and the P protein for that matter) was conspicuous by its absence, which suggests that both of those transport proteins function in the sorting pathway before melanosomes. Because only tyrosinase is secreted from OCA2 melanocytes, we hypothesize that the function of the P protein in the sorting pathway precedes that of MATP, as depicted in Fig. 6.

The uw-mutant melanocytes displayed a minimally pigmented melanosome population at stages I and II; these had irregular shapes and the internal fibers were not well organized (Fig. 5). Similar types of early immature melanosomes were seen in the secreted vesicle fraction. DOPA staining of uw-mutant melanocytes showed that active tyrosinase is localized in some of the early melanosomes of those cells. This ultrastructural phenotype of uw-mutant melanocytes is in good agreement with an earlier study (Lehman et al., 2000), which reported similar features of melanosomes in cultured melanocytes derived from mice carrying the uw^d (underwhite dense) mutation. The reduced melanin content and the irregular shape of melanosomes in melanocytes derived from mice carrying different mutations of the *uw* gene have been observed by others (Sweet et al., 1998; Lehman et al., 2000) and could also be seen in the eyes of those mice. This phenotype is strikingly similar to that previously reported for OCA2 melanocytes carrying mutations in the *p* gene (Puri et al., 2000).

In conclusion, we show in this study that MATP plays a crucial role in modulating the processing and intracellular trafficking of tyrosinase and other melanosomal proteins. The *uw* mutation is associated with a disruption of this pathway, which results in the misrouting and abnormal release of tyrosinase and Tyrp1 into the medium. Because tyrosinase is the key rate-limiting enzyme of melanogenesis, this results in the hypopigmented phenotype of uw-mutant melanocytes. The similarity of functions of the P protein and MATP, both being predicted transport proteins, in the trafficking of melanosomal proteins, provides valuable clues as to the roles of such proteins in regulating mammalian pigmentation.

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References

Basrur, V., Yang, F., Kushimoto, T., Yasumoto, K., Muller, J., Vieira, W. D., Watabe, H., Hearing, V. J. and Appella, E. (2003). Proteomic analysis of early melanosomes: Identification of novel melanosomal proteins. *J. Prot. Res.* **2**, 69-79.

Baxter, L. L. and Pavan, W. J. (2002). The oculocutaneous albinism type IV gene *Matp* is a new marker of pigment cell precursors during mouse embryonic development. *Mech. Dev.* **116**, 209-212.

Boissy, R. E., Zhao, H., Oetting, W. S., Austin, L. M., Wildenberg, S. C., Boissy, Y. L., Zhao, Y., Sturm, R. A., Hearing, V. J., King, R. A. et al. (1996). Mutation in and lack of expression of tyrosinase related protein 1 (TRP1) in melanocytes from an individual with brown oculocutaneous albinism: a new subtype of albinism classified as OCA3. *Am. J. Hum. Gen.* **58**, 1145-1156.

Chen, K., Manga, P. and Orlow, S. J. (2002). The pink-eyed dilution protein controls the processing of tyrosinase. *Mol. Biol. Cell* **13,** 1953-1964.

Halaban, R., Svedine, S., Cheng, E., Smicun, Y., Aron, R. and Hebert, D. N. (2000). Endoplasmic reticulum retention is a common defect associated with tyrosinase-negative albinism. *Proc. Natl. Acad. Sci. USA* **97**, 5889-5894.

Hearing, V. J. and Ekel, T. M. (1976). Mammalian tyrosinase: A comparison of tyrosine hydroxylation and melanin formation. *Biochem. J.* **157**, 549-557.

Hearing, V. J. and Tsukamoto, K. (1991). Enzymatic control of pigmentation in mammals. *FASEB J.* **5**, 2902-2909.

Huizing, M., Boissy, R. E. and Gahl, W. A. (2002). Hermansky-Pudlak syndrome: Vesicle formation from yeast to man. *Pigment Cell Res.* **15**, 405-419.

Jiménez, M., Maloy, W. L. and Hearing, V. J. (1989). Specific identification of an authentic clone for mammalian tyrosinase. *J. Biol. Chem.* **264**, 3397-3403.

Kikuchi, A., Shimizu, H. and Nishikawa, T. (1996). Expression and ultrastructural localization of HMB-45 antigen. *Br. J. Dermatol.* **135**, 400-405.

King, R. A. (1998). Albinism. In *The Pigmentary System: Physiology and Pathophysiology* (eds J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King and J. P. Ortonne), pp. 553-575. New York: Oxford University Press.

King, R. A., Oetting, W. S., Creel, D. and Hearing, V. J. (2002). Abnormalities of pigmentation. In *Emery and Rimoin's Principles and Practice of Medical Genetics* (eds D. L. Rimoin, J. M. Connor and R. E. Pyeritz,), pp. 3731-3785. New York: Churchill Livingstone.

Kobayashi, T., Urabe, K., Orlow, S. J., Higashi, K., Imokawa, G., Kwon, B. S., Potterf, S. B. and Hearing, V. J. (1994). The pmel17/*silver* locus protein: characterization and investigation of its role in mammalian melanogenesis. *J. Biol. Chem.* **269**, 29198-29205.

Kushimoto, T., Basrur, V., Matsunaga, J., Vieira, W. D., Muller, J., Appella, E. and Hearing, V. J. (2001). A new model for melanosome biogenesis based on the purification and mapping of early melanosomes. *Proc. Natl. Acad. Sci. USA* **98**, 10698-10703.

Lehman, A. L., Silvers, W. K., Puri, N., Wakamatsu, K., Ito, S. and Brilliant, M. H. (2000). The underwhite (*uw*) locus acts autonomously and reduces the production of melanin. *J. Invest. Dermatol.* **115**, 601-606.

Manga, P. and Orlow, S. J. (1999). The pink-eyed dilution gene and the molecular pathogenesis of tyrosinase-positive albinism (OCA2). *J. Dermatol.* **26**, 738-747.

Manga, P., Boissy, R. E., Pifko-Hirst, S., Zhou, B. K. and Orlow, S. J. (2001). Mislocalization of melanosomal proteins in melanocytes from mice with oculocutaneous albinism type 2. *Exp. Eye Res.* **72**, 695-710.

Manga, P., Kromberg, J. G., Box, N. F., Sturm, R. A., Jenkins, T. and Ramsay, M. (1997). Rufous oculocutaneous albinism in southern African Blacks is caused by mutations in the TYRP1 gene. *Am. J. Hum. Gen.* **61**, 1095-1101.

Negroiu, G., Branza-Nichita, N., Petrescu, A. J., Dwek, R. A. and Petrescu, S. M. (1999). Protein specific N-glycosylation of tyrosinase and tyrosinase-related protein-1 in B16 mouse melanoma cells. *Biochem. J.* **344**, 659-665.

Newton, J. M., Cohen-Barak, O., Hagiwara, N., Gardner, J. M., Davisson, M. T., King, R. A. and Brilliant, M. H. (2001). Mutations in the human orthologue of the mouse *underwhite* gene (*uw*) underlie a new form of oculocutaneous albinism, OCA4. *Am. J. Hum. Gen.* **69**, 981-988.

Oetting, W. S. and King, R. A. (1999). Molecular basis of albinism: mutations and polymorphisms of pigmentation genes associated with albinism. *Hum. Mutat.* **13**, 99-115.

Potterf, S. B., Furumura, M., Sviderskaya, E. V., Santis, C., Bennett, D. C. and Hearing, V. J. (1998). Normal tyrosine transport and abnormal tyrosinase routing in *pinkeyed-dilution* melanocytes. *Exp. Cell Res.* **244**, 319-326.

Puri, N., Gardner, J. M. and Brilliant, M. H. (2000). Aberrant pH of melanosomes in pink-eyed dilution (p) mutant melanocytes. *J. Invest. Dermatol.* **115**, 607-613.

Sarangarajan, R., Budev, A., Zhao, Y., Gahl, W. A. and Boissy, R. E. (2001). Abnormal translocation of tyrosinase and tyrosinase-related protein 1 in cutaneous melanocytes of Hermansky-Pudlak syndrome and in melanoma cells transfected with anti-sense *HPS1* cDNA. *J. Invest. Dermatol.* **117**, 641-646.

Schaumburg-Lever, G., Metzler, G. and Kaiserling, E. (1991). Ultrastructural localization of HMB-45 binding sites. *J. Cutan. Pathol.* **18**, 432-435.

Siegrist, W. and Eberle, A. N. (1986). In situ melanin assay for MSH using mouse B16 melanoma cells in culture. *Anal. Biochem.* **159**, 191-197.

Spritz, R. A. and Hearing, V. J. (1994). Genetic disorders of pigmentation. In *Advances in Human Genetics* (eds K. Hirschhorn and H. Harris), pp. 1-45. New York: Plenum Press.

Spritz, R. A., Strunk, K. M., Giebel, L. B. and King, R. A. (1990). Detection of mutations in the tyrosinase gene in a patient with Type IA oculocutaneous albinism. *New Eng. J. Med.* **322**, 1724-1728.

Sweet, H. O., Brilliant, M. H., Cook, S. A., Johnson, K. R. and Davisson, M. T. (1998). A new allelic series for the underwhite gene on mouse chromosome 15. *J. Hered.* **89**, 546-551.

Tamura, A., Halaban, R., Moellmann, G. E., Cowan, J. M., Lerner, M. R. and Lerner, A. B. (1987). Normal human melanocytes in culture. *In Vitro Cell Dev. Biol.* **23**, 519-522.

Toyofuku, K., Wada, I., Spritz, R. A. and Hearing, V. J. (2001a). The molecular basis of oculocutaneous albinism type 1 (OCA1): Sorting failure and degradation of mutant tyrosinase results in a lack of pigmentation. *Biochem. J.* **355**, 259-269.

Toyofuku, K., Wada, I., Valencia, J. C., Kushimoto, T., Ferrans, V. J. and Hearing, V. J. (2001b). Oculocutaneous albinism (OCA) types 1 and 3 are ER retention diseases: Mutations in tyrosinase and/or Tyrp1 influence the maturation, degradation of calnexin association of the other. *FASEB J.* **15**, 2149-2161.

Toyofuku, K., Valencia, J. C., Kushimoto, T., Costin, G. E., Virador, V. M., Vieira, W. D., Ferrans, V. J. and Hearing, V. J. (2002). The etiology of oculocutaneous albinism (OCA) type II: the pink protein controls the transport and processing of tyrosinase. *Pigment Cell Res.* **15**, 217-224.

Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. M. and Hearing, V. J. (1992). A second tyrosinase-related protein, TRP2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J.* **11**, 519-526.

Valverde, P., García-Borrón, J. C., Solano, F. and Lozano, J. A. (1992). Proteolysis with trypsin of mammalian tyrosinase isoforms from B16 mouse melanoma. *Arch. Biochem. Biophys.* **297**, 221-227.