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Genetics

The ocular albinism type 1 gene product is a membrane glycoprotein localized to melanosomes

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ABSTRACT **Ocular albinism type 1 (OA1) is an inherited disorder characterized by severe reduction of visual acuity, photophobia, and retinal hypopigmentation. Ultrastructural examination of skin melanocytes and of the retinal pigment epithelium reveals the presence of macromelanosomes, suggesting a defect in melanosome biogenesis. The gene responsible for OA1 is exclusively expressed in pigment cells and encodes a predicted protein of 404 aa displaying several putative transmembrane domains and sharing no similarities with previously identified molecules. Using polyclonal antibodies we have identified the endogenous OA1 protein in retinal pigment epithelial cells, in normal human melanocytes and in various melanoma cell lines. Two forms of the OA1 protein were identified by Western analysis, a 60-kDa glycoprotein and a doublet of 48 and 45 kDa probably corresponding to unglycosylated precursor polypeptides. Upon subcellular fractionation and phase separation with the nonionic detergent Triton X-114, the OA1 protein segregated into the melanosome-rich fraction and behaved as an authentic integral membrane protein. Immunofluorescence and immunogold analyses on normal human melanocytes confirmed the melanosomal membrane localization of the endogenous OA1 protein, consistent with its possible involvement in melanosome biogenesis. The identification of a novel melanosomal membrane protein involved in a human disease will provide insights into the mechanisms that control the cell-specific pathways of subcellular morphogenesis.**

The synthesis of melanin represents the main biochemical function of the pigment cells and is confined within specialized membrane-bound subcellular organelles, termed melanosomes (for review see refs. 1–3). Melanogenesis is regulated at several levels. In the mouse, for example, more than 65 distinct genetic loci have been identified that are involved in the control of coat color (4). In humans, inherited defects of melanogenesis result in a clinical condition called albinism, which comprises a heterogeneous group of diseases characterized by congenital hypopigmentation. This can be generalized, as in oculocutaneous albinism (OCA), or primarily localized to the eye, as in ocular albinism (1).

The molecular dissection of albinism has led to the identification of a number of genes involved in the process of melanin synthesis, such as tyrosinase and P, the mutations of which result in OCA1 and OCA2, respectively (1, 5–7). However, little is known about the pathogenesis of other types of albinism in which melanosome biogenesis, rather than the melanin synthetic pathway, appears to be primarily affected (8, 9). Organelle abnormalities can be restricted to melanosomes within melanocytes, as in ocular albinism type 1 (OA1), or can be shared by other intracellular organelles within many cell types, as in Hermansky–Pudlak syndrome and in Chediak–Higashi syndrome (1, 9, 10). In OA1, the presence of macromelanosomes, also referred to as melanin macroglobules, represents a constant and specific histological finding in the melanocytes of the skin and in the retinal pigment epithelium (8, 11–13). Patients with OA1 have classical ocular features of albinism, including severe reduction of visual acuity, retinal hypopigmentation, foveal hypoplasia, iris translucency, and misrouting of the optic tracts, but they have normal cutaneous pigmentation (1, 8). This finding can be explained by a higher sensitivity of the optic system to the melanogenesis disorders, particularly during development.

Recently we have identified the OA1 gene which is expressed at high levels only in RNA samples from retina, including the retinal pigment epithelium, and from melanoma (14). The OA1 gene encodes a predicted protein product of 404 aa displaying several putative transmembrane domains and sharing no similarities with previously identified molecules (14). Mutation analysis of the OA1 gene coding region revealed the presence of several functionally significant mutations. As a first step toward understanding the role of the OA1 protein in melanosome morphogenesis, we now report the identification of the endogenous OA1 protein product in retinal pigment epithelial cells and in melanocytic cells, including normal human melanocytes, its processing and behavior as an integral membrane protein, and its localization to melanosomes.

METHODS

Cell Culture and Transfection. Normal human melanocytes were isolated and cultivated from *in vitro* reconstituted epidermal sheets as described (15–17). After purification melanocytes were cultivated in DMEM and Ham's F-12 media (3:1 mixture) or E-199 containing fetal calf serum (10%), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), epidermal growth factor (10 ng/ml), basic fibroblast growth factor (1 ng/ml), glutamine (4 mM), and penicillin-streptomycin (50 units/ml). Melanocytes were isolated and cultivated either in the presence or in the absence of phorbol 12-myristate 13-acetate (PMA; 10 ng/ml). After their purification (100% positive to dopa reaction), melanocytes were used in experiments.

All cell lines were grown in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 200 units of penicillin-streptomycin per ml. RPEmsma were obtained from K. Dutt (Morehouse School of Medicine, Atlanta), gMel were kindly

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Abbreviations: OCA, oculocutaneous albinism; OA1, ocular albinism type 1; LGF, large granule fraction; PMA, phorbol 12-myristate 13-acetate; TRP, tyrosinase-related protein.

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provided by S. J. Orlow (New York University School of Medicine), and M14 were obtained by C. Rugarli (S. Raffaele Hospital and Milan University School of Medicine, Milan, Italy).

The OA1 expression vector pRH4/8 was obtained by subcloning the cDNA insert from clone AR16H (14), which contains the entire coding region, into the *Hin*dIII–*Xba*I sites of the expression vector pRc/RSV (Invitrogen). Cell transfections were performed by using the Lipofectamine reagent (GIBCO/BRL) according to the manufacturer's instructions.

Northern Blot Analysis. Cytoplasmic RNA from human melanocytes and from different cell lines was prepared according to standard procedures (18). Northern blot analysis was performed following standard methods (18). High stringency washing conditions were used (0.5× standard saline citrate/0.1% SDS at 65°C).

Production of Polyclonal Antibodies. The fusion protein CIII3 was generated by cloning the 3′ portion of the OA1 cDNA coding region (corresponding to amino acids 287–401 of the predicted protein) (14) into the pQE-30 vector (QIAexpressionist System; Quiagen, Chatsworth, CA) and purified according to the manufacturer's instructions. A 14-aa carboxyl-terminus peptide (14) conjugated with ovalbumin was obtained by PRIMM (Milan). Rabbit polyclonal antibodies were generated against both the fusion protein CIII3 (W7 and X7 antisera) and the synthetic peptide (Y7 and Z7 antisera) by PRIMM. All antibodies were immunoaffinity purified using the ImmunoPure Ag/Ab Immobilization Kit (Pierce) according to the manufacturers' instructions.

Subcellular Fractionation and Western Immunoblotting Analysis. Cultured melanocytes and cell lines were homogenized in cold homogenization buffer (0.25 M sucrose/50 mM phosphate buffer, pH 6.8/1 mM EDTA/5 mM benzamidine/4 μg of aprotinin per ml/2 μg of leupeptin per ml/2 μg of pepstatin per ml) and the melanosome-rich large granule fraction (LGF) was isolated as described (19–21). Following solubilization of the LGF pellet in 1% Triton X-114, 0.1 M phosphate buffer (pH 6.8), 20 μg of aprotinin per ml, and 20 μg of leupeptin per ml, the aqueous and detergent phases were separated as described (21–23). For glycosylation analysis, protein samples (0.5–1 μg/μl) were digested overnight with 0.02 unit of *N*-glycosidase F per ml (Boehringer Mannheim) according to the manufacturers' instructions.

Western immunoblotting analysis was performed following standard procedures (24). Before loading, protein samples were mixed with equal volume of loading buffer containing 14% SDS and warmed at 37°C for 5 min. Lower SDS concentrations or boiling were usually avoided due to the precipitation of the OA1 protein under these conditions. Primary antibodies were used at 0.1 μg/ml (W7) to 0.3 μg/ml (X7, Y7, Z7) of specific immunoglobulins. Visualization of antibody binding was carried out with Enhanced ChemiLuminescence (Amersham) according to the manufacturer's instructions.

Cryoimmunoelectronmicroscopy. Pellets of human melanocytes were fixed in 3.7 paraformaldehyde in 0.2 M Hepes buffer (pH 7.4) for 5 min at room temperature, infiltrated in 2.1 M sucrose in phosphate-buffered saline (PBS), and immediately frozen in liquid nitrogen. Sections (60–80 nm) were cut either with tungsten-coated glass knives or with a diamond knife (Diatome), picked up by a drop of 2.3 M sucrose, and laid on formvar coated 200 mesh nickel grids. The OA1 protein was localized by immunogold techniques using the IgG fraction of the W7 antiserum (20 μg/ml) or, alternatively, the immunoaffinity purified preparation of the same antiserum (1.6 μg/ml). All antibodies and immunoreagents were diluted in PBS/glycine, and incubations were performed at room temperature. The second step reagents were either 20 nm gold-labeled protein A, 1:20 (ICN) for 30 min or a sequence of mouse anti-rabbit IgG, 1:20 (Dako) for 30 min, followed by 10 nm gold-labeled goat anti-mouse IgG, 1:50 (ICN) for 30 min. The sections were then stained on a drop of 3% uranyl acetate (Polysciences), diluted 1:10 in 2% solution of 25 centipoise methylcellulose (Methocel; Fluka) for 15 min, and air dried overnight before being observed and photographed with a Zeiss EM 10/C electron microscope.

RESULTS

Identification of the Protein Product of the OA1 Gene. Polyclonal antisera were generated against a recombinant protein expressed in *Escherichia Coli* (CIII3) corresponding to the last 114 aa of the protein product predicted from the OA1 gene sequence (14). This region was chosen because it contained the largest hydrophilic region of the OA1 protein. Antisera were also raised against a 14-aa peptide corresponding to the carboxyl terminus. Both anti-CIII3 (W7 and X7) and antipeptide (Y7 and Z7) antisera specifically recognized the original antigen in dot blot assays, with different titers. All antisera were purified by affinity chromatography and used in Western blotting analysis of protein extracts from human melanocyte primary cultures and from several established cell lines, including COS-7 (monkey kidney epithelial cells), HeLa (human transformed cervical epithelial cells), RPEmsma (human retinal pigment epithelial cells), gMel (human pigmented melanoma cells), and M14 (human unpigmented melanoma cells) (Fig. 1). A broadly migrating major band of 60 kDa together with a doublet of 48–45 kDa were identified only in extracts from cells which were previously shown to express the OA1 mRNA (Fig. 1). In most experiments, the 60-kDa band behaved as a tight doublet. The presence of doublets instead of single bands could indicate the presence of minor post-translational modifications or of partial digestion products, in spite of the use of antiproteases. The same bands were observed with all four antisera and were absent in Western blots performed with preimmune sera. The reactivity of W7 and X7 antisera were blocked by competition with the recombinant protein CIII3 used for the immunizations (data not shown). Transfection of COS-7 and HeLa cells with the OA1 expression vector pRH4/8 (see *Methods*) resulted in a strong expression of a recombinant protein corresponding to the doublet of 48 and 45 kDa (see below and Fig. 3).

Subcellular Segregation and Processing of the OA1 Protein. The subcellular segregation, processing and fine intracellular localization of the OA1 protein were analyzed in normal human melanocytes. Subcellular fractionation of normal human melanocytes showed that the 60-kDa and the 48- and 45-kDa proteins are enriched in the 10,000 × *g* pellet (Fig. 2), corresponding to the LGF, in which all stages of melanosomes are recovered (19). After Triton X-114 solubilization and phase separation of the 10,000 × *g* pellet, the 60-kDa and the 48- and 45-kDa proteins segregated into the detergent phase, behaving as authentic integral membrane proteins (22). This finding is consistent with the hydrophobic profile of the predicted OA1 protein which suggests the presence of at least six transmembrane domains in the OA1 protein (25).

Several melanosomal membrane proteins, and particularly tyrosinase and the tyrosinase-related proteins (TRPs) are glycosylated (26–28). The amino acid sequence of the predicted OA1 protein reveals the presence of two putative *N*-glycosylation sites. *N*-glycosidase F digestion of cellular extracts from normal human melanocytes resulted in the disappearance of the 60-kDa band, and in a more intense signal corresponding to the 48- and 45-kDa bands (Fig. 3). This indicates that the 60-kDa band is a glycosylated protein and the 48- and 45-kDa bands probably represent precursor polypeptides. However, when the OA1 expression vector pRH4/8 (see *Methods*) was introduced by lipofection in nonmelanocytic cells such as COS-7 and HeLa, the 60-kDa glycoprotein was not produced, and a doublet corresponding to the 48- and 45-kDa polypeptides was seen both in the presence and in the

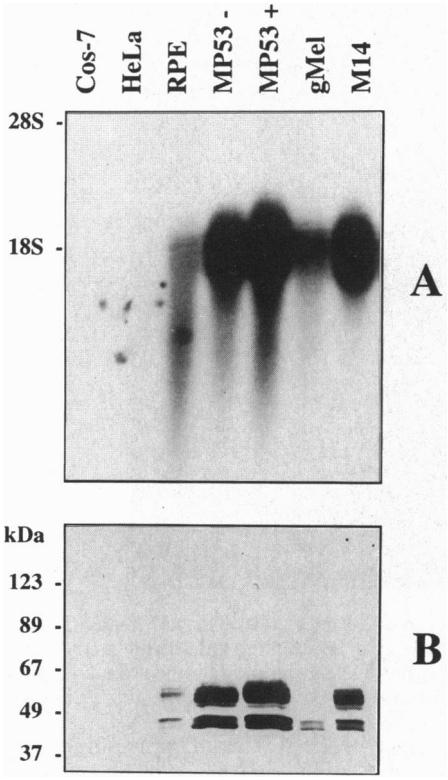

FIG. 1. Expression analysis of the OA1 mRNA and protein in various cell types: COS-7, monkey kidney epithelial cells; HeLa, human transformed cervical epithelial cells; RPE, human retinal pigment epithelial cells; MP53, human melanocytes cultured in the absence (−) or in the presence (+) of PMA; gMel, human pigmented melanoma cells and M14, human unpigmented melanoma cells. (*A*) Hybridization of cDNA clone AR16H to a Northern blot containing 20 μg of total RNA from each cell type. A single 1.65-kb transcript is detected only in retinal pigment epithelial cells and in melanocytic cells, in agreement with previously reported data (14). (*B*) For Western blotting analysis, 20 μg of Triton X-114 soluble proteins from the LGF of each cell type were subjected to SDS/PAGE followed by immunoblot with the X7 antiserum (used at 0.3 μg/ml of specific immunoglobulins). A broadly migrating band of 60 kDa and a doublet of 48 and 45 kDa are identified in retinal pigment epithelial cells and in melanocytic cells, consistent with the Northern results. In the gMel cells the 60-kDa glycoprotein is visible upon overloading of the gel, albeit less represented than the 48- and 45-kDa proteins.

absence of *N*-glycosidase F treatment (Fig. 3). In addition, a complex pattern of aberrant highly glycosylated forms, which are converted to the precursor 48- and 45-kDa polypeptides upon *N*-glycosidase F digestion, was observed, particularly in transfected HeLa cells. These data suggest that glycosylation of the OA1 polypeptide is controlled by cell-specific pathways in retinal pigment epithelial and melanocytic cells.

In addition to the 60-, 48-, and 45-kDa bands, in cellular extract from normal human melanocytes the X7 and W7 antisera (but not Y7 and Z7 antisera) recognized a 90-kDa protein, which behaved as a melanosomal matrix protein upon subcellular fractionation (see Fig. 2), and needed the addition of SDS to 1% for effective solubilization. Western blotting analysis of the Triton X-114 insoluble fractions from various cell lines revealed that the 90-kDa protein is present, although in little amounts, in the cellular extracts from the cell lines that were previously shown to express the OA1 mRNA (data not shown). On the contrary, the 90-kDa band was absent in the cellular extracts from COS-7 and HeLa, and from a melanoma cell line not expressing the OA1 mRNA (data not shown). Although the precise nature of the 90-kDa protein remains to be clarified, preliminary data suggest that it represents a highly

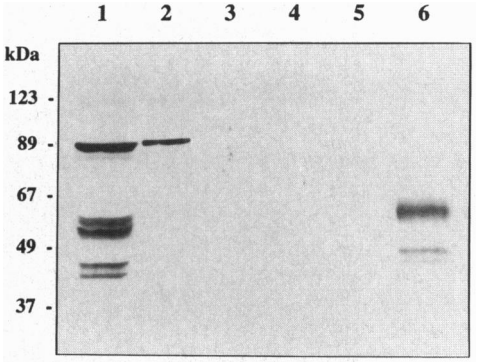

FIG. 2. Subcellular distribution of the OA1 protein. Human melanocytes cultured in presence of PMA were subjected to subcellular fractionation. Protein samples from each fraction were separated by SDS/PAGE, followed by immunoblot analysis with the X7 antiserum (used at 0.3 μg/ml of specific immunoglobulins). Lanes: 1, total cellular extract (20 μg); 2, LGF, Triton X-114 insoluble fraction (5 μg); 3, 10,000 × *g* supernatant, Triton X-114 aqueous phase (2.5 μg); 4, 10,000 × *g* supernatant, Triton X-114 detergent phase (2.5 μg); 5, LGF, Triton X-114 aqueous phase (2.5 μg); 6, LGF, Triton X-114 detergent phase (2.5 μg). The 60-kDa and 48- and 45-kDa bands segregate in the Triton X-114 detergent phase, as expected for authentic integral membrane proteins, while the 90-kDa band remains insoluble in Triton X-114.

glycosylated derivative of the 48- and 45-kDa precursor polypeptides.

Fine Subcellular Localization of the OA1 Protein. Immunofluorescence analysis on melanocytic cells was performed using antisera W7 and X7, which strongly recognized the recombinant OA1 protein in transfected COS-7 cells, while untransfected cells were negative (data not shown). These antisera were used for the analysis of RPEmsma (human retinal pigment epithelial cells), gMel (human pigmented melanoma cells), M14 (human unpigmented melanoma cells), and normal human melanocytes. In these cells, the W7 and X7 antisera showed a granular cytoplasmic reactivity, more concentrated in the perinuclear area and spread toward the periphery (Fig. 4). A similar pattern of subcellular distribution in melanocytic cells was observed with the monoclonal antibody to the melanosomal protein Pmel 17 (HMB45) (refs. 20,

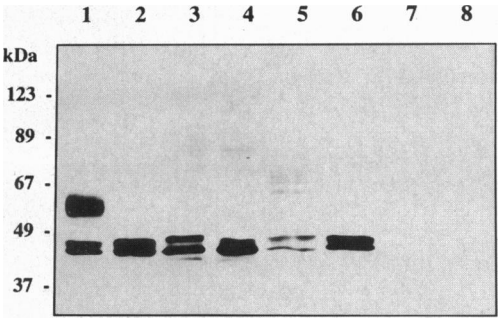

FIG. 3. Glycosylation of the OA1 protein. Whole cell pellets were solubilized in *N*-glycosidase F buffer containing 1% Nonidet P-40 and subjected to *N*-glycosidase F digestion (see *Methods*). The same amount of proteins were separated on SDS/PAGE, followed by immunoblot analysis with the X7 antiserum (used at 0.3 μg/ml of specific immunoglobulins). Lanes: 1, human melanocytes; 2, human melanocytes after *N*-glycosidase F treatment; 3, COS-7 cells transfected with the pRH4/8 expression vector; 4, same as lane 3 after *N*-glycosidase F treatment; 5, HeLa cells transfected with the pRH4/8 expression vector; 6, same as lane 5 after *N*-glycosidase F treatment; 7, COS-7 cells; 8, HeLa cells. A complex pattern of aberrant highly glycosylated forms, which are converted to the precursor 48- and 45-kDa polypeptides upon *N*-glycosidase F treatment, is observed, particularly in transfected HeLa cells.

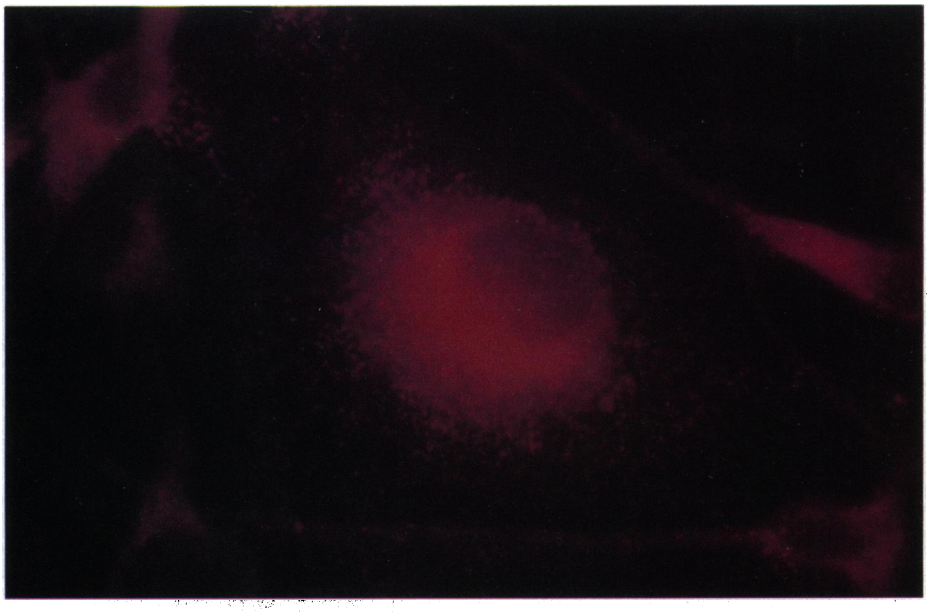

FIG. 4. Immunofluorescence analysis of the OA1 protein. Normal human melanocytes were fixed 5–10 min with methanol at −20°C and incubated with antibody X7 at 1.5 μg/ml of specific immunoglobulins. Tetramethylrhodamine B isothiocyanate-conjugated swine anti-rabbit immunoglobulins (Dako) were used as secondary antibody. The anti-OA1 antiserum shows a perinuclear cytoplasmic and granular reactivity, spread toward the periphery. (×1000.) In paraformaldehyde-fixed samples a similar pattern was observed, albeit with a marked reduction in signal intensity.

23, and 29–31; data not shown) and has been described for other well-characterized melanosomal proteins, such as the TRPs (32–34).

The ultrastructural localization of the OA1 protein was studied using the W7 antiserum in an immunogold technique on ultrathin cryosections of cultured normal human melanocytes. Most melanosomes within these cells were at stage III and IV of maturation (Fig. 5), while about 20% of melanosomes were at stage II (Fig. 5*a*). Approximately 30% of melanosomes per cell profile showed a gold particle labeling the outer surface of the surrounding membrane (Fig. 5 *b–d*). In a few instances gold particles were also observed in the interior of the organelle at all stages of maturation (Fig. 5*a*). Besides the melanosomes, gold labeling was observed within rough endoplasmic reticulum cisternae (Fig. 5*b*) and clustered in groups of 3–4 within light, membrane-bound areas of the cytoplasm (data not shown), possibly representing stage I melanosomes. The same results were obtained with two different antibody preparations and two different system of gold-labeled second reagents.

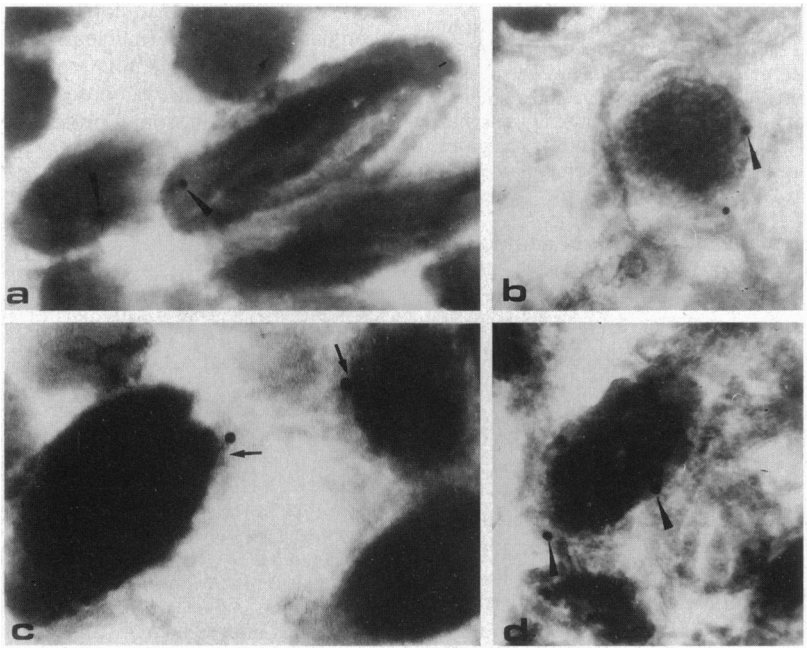

FIG. 5. Electron micrograph of melanosomes in cultured human melanocytes. (*a*) Gold particles (arrowheads) label the matrix of two melanosomes, namely a stage IV on the left and a late stage II on the right. (×91,000.) (*b*) A gold particle (arrowhead) labels the surface of a stage III melanosome, another gold particle labels an elongated rough endoplasmic reticulum cistern. (×80,000.) (*c*) Two stage IV melanosomes gold labeled on the surface, the arrows indicate the surrounding membrane. (×125,000.) (*d*) Another stage IV melanosome gold labeled on the surface (arrowheads). (×84,600.) The observation that only 30% of the melanosomes show a gold labeling, and that only one or a few gold particles labeled each melanosome, can be explained by the tested reduction in antigen binding shown by the W7 antiserum after paraformaldehyde fixation in immunofluorescence analysis. However, we cannot exclude that only a few copies of the OA1 protein are present per melanosome.

DISCUSSION

The OA1 gene was isolated using a positional cloning strategy, based exclusively on the position of the gene on the X chromosome and on the identification of mutations in patients (14). As the OA1 protein does not share similarities with previously described molecules, it is difficult to predict its function. However, the presence of macromelanosomes in the melanocytes and the retinal pigment epithelium of patients with OA1 (8, 11–13) and the presence of several hydrophobic domains suggest that the product of the OA1 gene could be a membrane protein which is essential for the formation and/or maturation of melanosomes.

By using several antisera directed against the predicted OA1 protein, we have identified a 60-kDa band and a doublet of 48- and 45-kDa in human retinal pigment epithelial cells, normal human melanocytes and human melanoma cells, but not in COS-7 and HeLa cells, in complete agreement with the mRNA expression data. *N*-glycosidase F treatment of the protein extracts resulted in the conversion of the 60-kDa band into the doublet of 48 and 45 kDa, the former being interpreted as the glycosylated form of the latter. The 48- and 45-kDa polypeptides, which show a size consistent with the expected molecular mass of the OA1 protein backbone inferred from the primary amino acid sequence, were considered as precursors.

Transient expression of the OA1 cDNA in COS-7 and HeLa cells resulted in the production of the 48- and 45-kDa proteins, providing further evidence that we have identified the protein product of the OA1 gene. However, neither COS-7 nor HeLa cells were able to process the 48- and 45-kDa proteins to the 60-kDa glycoprotein; moreover, this capability appeared to be strongly reduced in a pigmented melanoma cell line (gMel; see Fig. 1). In COS-7 and HeLa cells glycosylation seems not to be abolished, but the protein does not follow the same processing pathway as in melanocytic cells (Fig. 3). A possible explanation for these results is the presence of melanocytic cell-specific pathways by which the endogenous OA1 protein is converted into the 60-kDa glycoprotein, and which have been partially lost in the gMel cell line in the course of neoplastic transformation.

Subcellular fractionation showed that the OA1 protein localizes into the melanosome-rich LGF, is soluble in the nonionic detergent Triton X-114 and segregates into the detergent phase upon phase separation, as expected for an authentic integral membrane protein (22). These results confirm the hydrophobic nature of the OA1 protein suggested by the analysis of the predicted amino acid sequence (14).

Melanosomes are the pigment cell-specific subcellular organelles in which the synthesis of melanin is confined. A number of melanosomal proteins involved in the control of melanogenesis in mice and humans have been identified. These include tyrosinase, TRP-1 and TRP-2, which represent melanosomal membrane enzymes involved in the melanin biosyntetic pathway, the product of the P gene, an integral membrane protein for which a tyrosine transport function has been hypothesized, and the Pmel 17 protein, a putative melanosomal matrix protein (20, 21, 33, 35–38). Immunofluorescence analysis of melanocytic cells using the anti-OA1 antiserum showed a pattern of subcellular distribution similar to that observed for other melanosomal proteins, including Pmel 17 and TRPs (refs. 23, 30, and 32–34; data not shown). Immunogold analysis of normal human melanocytes definitively assigned the OA1 protein to the membrane of melanosomes at all stages of maturation. However, a minority of gold particles was observed in the interior of the organelles suggesting that the OA1 protein itself, or another protein sharing antigenic homologies, may be shed or remain engulfed within the melanosome lumen.

Melanocytes from OA1 patients show structural abnormalities of melanosomes, mainly represented by macromelanosomes, suggesting that the OA1 protein function could be related to the organelle biogenesis process (8, 11, 12). The finding of macromelanosomes is not unique to OA1, as they have been described in a number of other conditions, such as neurofibromatosis, xeroderma pigmentosum, naevus spilus, multiple lentigo, Albright's syndrome, Chediak–Higashi syndrome, and Hermansky–Pudlak syndrome (8, 10–12).

Both Chediak–Higashi syndrome and Hermansky–Pudlak syndrome result from unknown primary defects affecting the morphogenesis of subcellular organelles in several cell types. These include melanosomes within melanocytes, storage granules within platelets, and lysosomes within reticuloendothelial cells and leucocytes. The notion that melanosomes and lysosomes could derive from the same endosomal pathway of morphogenesis has been supported by several lines of evidence (28, 34, 39, 40). It is possible that the OA1 protein function is necessary only for melanosome formation, while the products of the genes involved in Chediak–Higashi syndrome and Hermansky–Pudlak syndrome act upstream in a common biogenesis pathway shared by different subcellular organelles (9). However, we cannot exclude a different biological function for the OA1 protein, such as the transport of melanin metabolites in and out of melanosomes. Further studies are needed to clarify the role of the OA1 protein in the process which finally leads to proper melanosome differentiation, as well as its possible involvement and interaction with the other melanosomal proteins in the control of melanogenesis within the pigment cells.

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