The AP-3-dependent targeting of the melanosomal glycoprotein QNR-71 requires a di-leucine-based sorting signal

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SUMMARY

The Quail Neuroretina clone 71 gene (QNR-71) is expressed during the differentiation of retinal pigmented epithelia and the epidermis. It encodes a type I transmembrane glycoprotein that shares significant sequence homologies with several melanosomal proteins. We have studied its intracellular traffic in both pigmented and non-pigmented cells. We report that a di-leucine-based sorting signal (ExxPLL) present in the cytoplasmic domain of QNR-71 is necessary and sufficient for its proper targeting to the endosomal/premelanosomal compartments of both pigmented and non-pigmented cells. The intracellular transport of QNR-71 to these compartments is mediated by the AP-3 assembly proteins. As previously observed for the lysosomal glycoproteins LampI and LimpII, overexpression of QNR-71 increases the amount of AP-3 associated with membranes, and inhibition of AP-3 synthesis increases the routing of QNR-71 towards the cell surface. In addition, expression of QNR-71 induces a misrouting of endogenous LampI to the cell surface. Thus, the targeting of QNR-71 might be similar to that of the lysosomal integral membrane glycoproteins LampI and LimpII. This suggests that sorting to melanosomes and lysosomes requires similar sorting signals and transport machineries.

Key words: AP-3, Retina, Glycoproteins, Sorting, Melanosome

INTRODUCTION

Melanosomes, usually considered as lysosome-like structures, are specialized organelles of melanocytes in which melanin is synthesized. Pigment synthesis requires the combined action of at least 85 gene products, including soluble and membrane proteins (Jackson, 1997; Hearing, 1999). During the past years, the heterotetrameric AP-3 adaptor complex, one of the four adaptor complexes involved in membrane traffic, has been involved in protein targeting to melanosomes (for a review see Le Borgne and Hoflack, 1998a; Lloyd et al., 1998; Odorizzi et al., 1998; Swank et al., 1998; Jimbow et al., 2000; Setaluri, 2000). Mutations in the Drosophila garnet, carmine or ruby genes, the orthologs of the mammalian genes encoding the δ, μ3 (Mullins et al., 1999; Ooi et al., 1997; Simpson et al., 1997) or β3 subunits of AP-3 (Kretzschmar et al., 2000) cause a reduction in the pigmentation of the eyes and other tissues. Similarly, the genes encoding the mouse β3A or δ-adaptin are altered in the mouse hypopigmentation mutants pearl and mocha, respectively (Feng et al., 1999; Kantheti et al., 1998; Zhen et al., 1999). Furthermore, the major melanogenic enzyme, tyrosinase, whose targeting to lysosomal compartments of non-pigmented cells involves a dileucine- and a tyrosine-based sorting motif (Calvo et al., 1999; Simmen et al., 1999), is able to interact with AP-3 in vitro (Höning et al., 1998). However, it is also known that AP-3 is involved in the intracellular targeting of lysosomal membrane glycoproteins such as lysosomal-associated membrane proteins (Lamps) and lysosomal integral membrane proteins (Limp3; Dell’Angelica et al., 1999; Le Borgne et al., 1998). Inhibition of the μ3 subunit synthesis (Le Borgne et al., 1998) or mutations in the human β3A subunit, as seen in some cases of Hermansky-Pudlak syndrome, an autosomal recessive disorder characterized by oculocutaneous albinism (Dell’Angelica et al., 1999), alter the normal trafficking of lysosomal membrane glycoproteins. This could potentially explain why in addition to hypopigmentation defects, the lysosomes and platelet-dense granules are also abnormal, leading to bleeding defects (Lloyd et al., 1998; Odorizzi et al., 1998; Swank et al., 1998). Altogether, these observations suggest that targeting to lysosomes and melanosomes share common sorting mechanisms. In pigmented cells, however, melanosomes contain a unique set of resident membrane proteins (King et al., 1995) that are distinguishable from that of the late endosomal/lysosomal membrane system (Raposo et al., 2001), indicating that the two types of proteins could be segregated from each other in the endocytic pathway (Orlow et al., 1993). Pigmented retina cells may provide a good system with which to study the biogenesis of melanosomes, in particular the sorting machineries responsible for transport of typical...
melanosomal proteins. Quail neuroretina (QNR) cells infected with the v-Myc-expressing retrovirus MC29 have been shown to become pigmented after several passages in vitro (Martin et al., 1992). A subsequent differential screening lead to the identification of a cDNA (QNR-71) selectively expressed in the pigmented layer of the retina and in the epidermis (Turque et al., 1996). The derived amino acid sequence revealed a significant homology with the product of the human PMEL17 (SILV) gene expected to be the equivalent of the mouse silver gene (Kwon et al., 1991), the chicken matrix melanosomal protein MMP115, a retinal pigmented epithelium specific protein (Mochii et al., 1991) or the product of human NMB (GP/NMB) gene, a gene expressed in low metastatic melanoma cell lines (Weterman et al., 1995). Thus, QNR-71 is likely to encode a melanosomal protein. The amino acid sequence indicates that it consists of a 22 hydrophobic amino acid long N-terminal signal sequence followed by a 464 amino acid long luminal domain containing 11 potential N-glycosylation sites, a single membrane spanning domain and a 50 amino acid long cytoplasmic domain. This latter contains a putative tyrosine-(YKPI) and a putative di-leucine-based (ExxPLL) sorting signal analogous to those found in many melanosomal proteins, and required for endosomal/lysosomal targeting (Kirchhausen et al., 1997; Le Borgne and Hoflack, 1998a; Le Borgne and Hoflack, 1998b; Mullman, 1996; Simmen et al., 1999).

Despite these sequence homologies, the intracellular distribution and the intracellular trafficking of QNR-71 remains unknown. In this study, we have expressed epitope-tagged versions of QNR-71 in pigmented and non-pigmented cells to determine its steady-state distribution. We show here that it localizes into peripheral endosomal/premelanosomal dotted structures in cells from the retinal pigmented epithelium (RPE) and to early and late endocytic compartments when ectopically expressed in HeLa cells. The di-leucine-based sorting motif is shown to be necessary and sufficient to mediate the transport of QNR-71 to these compartments. QNR-71 transport requires the AP-3 complex. Inhibition of AP-3 synthesis causes the misrouting of both endogenous LampI and QNR-71 to the cell surface. Moreover, ectopic expression of QNR-71 in HeLa cells is sufficient to reroute endogenous LampI to the plasma membrane. Together, these results suggest that melanosomal and lysosomal membrane glycoproteins share some aspects of membrane trafficking.

MATERIALS AND METHODS

Materials

All materials were of analytical grade. DOTAP reagent was from Boehringer Mannheim GmbH (Mannheim, Germany), PEI transfection reagent was from (Euromedex, Strasbourg, France). [35S] methionine/cysteine (EXPRESS mix) was from NEN Life Science Products. 30% (w/v) Acrylamide/0.8% (w/v) bis-acrylamide solution was from National Diagnostics (Atlanta, GA). Protein A sepharose was from Pharmacia Amersham.

Antibodies

The δ-subunit of AP-3 complex were decorated using an affinity purified rabbit polyclonal antibody (a kind gift from Dr M. S. Robinson, Cambridge) as previously described (Le Borgne et al., 1998). The 100/3 monoclonal antibody directed against γ-adaptin was from Sigma. Early endosomal-associated antigen 1 (EEA1) was decorated with a mouse monoclonal antibody (Transduction Laboratories, Lexington, KY). Human LampI was detected using the H4A3 mouse monoclonal antibody (Developmental Hybridoma Bank, Iowa, IA). Vesicular somatostatin virus-G protein (VSV-G) was detected with a rabbit polyclonal antibody (Alconada et al., 1996) or the PSD4 mouse monoclonal antibody (a kind gift of Thomas Kreis). Man 6-P/IGF II receptor was detected using a rabbit polyclonal antibody (Méresse and Hoflack, 1993). The anti gpl antibodies were as described previously (Alconada et al., 1996). All secondary antibodies against the Fc fragments of mouse and rabbit IgGs coupled to FITC or Texas Red were from Jackson Laboratories (Immunootech, Marseille, France).

Cell culture and transfections

Dissociated cells from the retina pigmented epithelium (RPE) dissected from 8-day-old quail embryos were plated on gelatin-coated dishes in Dulbecco's/F12 medium containing 10% fetal calf serum, 1% vitamin modified Eagle's medium 100X and 10% conalbumin (complete medium).

HeLa cells (American Type Tissue Culture Collection, Rockville, MD) were grown in α-minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and streptomycin. For transfection, cells were split and grown onto coverslips the day before. Transient transfections using the PEI reagent were performed as described by the manufacturer. Briefly, cells were transfected for 12 hours with the corresponding DNAs and allowed to express the chimeric proteins for 24 to 72 hours. Transient transfection using the recombinant vaccinia virus were performed as described previously (Le Borgne et al., 1998). Briefly, the cells were infected for 30 minutes with the vT7 recombinant virus and transfected for 1 hour with the different DNAs using the DOTAP reagent. The cells were allowed to express the different chimeric proteins for 2 to 6 hours in the presence of 5 mM hydroxyurea to avoid cytotoxic effects. Under these conditions, the bulk of the expressed proteins is still present in the perinuclear compartments (mostly the Golgi apparatus) and have not yet reached their final destination. Part of the expressed proteins is also present at the cell surface, owing to their overexpression (Figs 6, 7).

To inhibit the synthesis of the ubiquitously expressed μ3A chain of AP-3, a combination of two antisense or sense phosphorothioate-modified oligonucleotides were added to the cell culture medium at a final concentration of 10 μM for 48 hours as previously reported (Le Borgne et al., 1998).

Plasmid construction and mutagenesis

To generate the VSV-G-QNR-71 chimera, the VSV-G tag was first cloned into the unique Cell II site of the full-length wild-type QNR-71. The VSV-G tag was made by annealing the following primers: forward primer, 5'-TGAGCCCTTACCGAATCGAGATGAACAAGCCTGGGAAAGGAA TGCG3; reverse primer, 5'-CGGAATGTGGGCTGGGGAGAAGGATCCGGG3. The fragment was then digested with EcoRI/XhoI and cloned into the same sites of the pcDNA3 vector (Invitrogen, San Diego, CA).

The L551G, L552G, and Y514A mutated versions of QNR-71 were obtained by PCR using the Quick Change kit (Stratagene, La Jolla, CA) using the wild-type QNR-71 as a template and the following primers: for the L551G mutagenesis, 5'-CCTGAGAGAATTCCTGGATTGAAAAGCAAACCGAGCTC3; for the L552G mutagenesis, 5'-CGAGAGAAATTCCTGGGCAACAAGCCTGAC3; for the Y514A mutagenesis, 5'-CAAAGAGATAAAACAGCTAAGCCCTATTTGAGAGATC3. The fragment was then digested with EcoRI/XhoI and cloned into the same sites of the pcDNA3 vector (Invitrogen, San Diego, CA).

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For the gpl-QNR chimera, the following primers where used using the gpl-Δ1 as a PCR template: forward primer gpl-1 (Alconada et al., 1996); and reverse primer, 5'-AAAGCTTTAGCTTTTCAACAGAGG-
ATTTCTCAGTGCTTTAGGAAGAAAAGGCTTT3'. The resulting PCR product was then cloned into the XbaI and HindIII sites of the pSFFV or pGEM-1 vectors.

**Indirect immunofluorescence and image processing**

Cells were processed for immunofluorescence as previously described (Le Borgne et al., 1998) and observed using an axioplan 2 microscope (Zeiss, Jena, Germany) and a 63x/1.4 numerical aperture immersion oil lens. Images were captured using a cooled charged-coupled device (CCD; Micromax from Princeton Instruments; Trenton, NJ) that had a Kodak RTE/CCD-1317K/1 chip (grade 1) for 12 bit image collection and was controlled by the IpLab Spectrum (Signal Analytics Corp., Vienna). To quantify AP-3 recruitment, randomly chosen fields were captured using the Micromax camera. In every field, the δ-adaptin labeled areas from transfected and non-transfected cells were selected and the fluorescence intensity (mean intensity/pixel) was calculated using the IpLab Spectrum software.

**Pulse-chase experiments and immunoprecipitations**

Cells from the RPE or HEk cells grown on plastic were infected and transfected as mentioned above. They were then pulse labeled for 30 minutes with 0.2 mCi/ml of [35S] methionine/cysteine and chased for the indicated times. Cells were then lysed for 30 minutes on ice in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and benzamidine, 5 μg/ml aprotinin and 1 μg/ml leupeptin) and spun for 15 minutes in an Eppendorf centrifuge. After a pre-clearing with a preimmune rabbit serum, samples were incubated with the indicated antibodies overnight at 4°C, spun for 15 minutes, and incubated for 1 hour with protein A-Sepharose. After washes, the immune complexes were collected using a cooled centrifuge. After a pre-clearing with a preimmune rabbit serum, samples were incubated with the indicated antibodies overnight at 4°C, spun for 15 minutes, and incubated for 1 hour with protein A-Sepharose. After washes, the immune complexes were resolved on 10% SDS-PAGE followed by fluorography.

**RESULTS**

**Localization of QNR-71 in pigmented and non pigmented cells**

The primary sequence of QNR-71 suggests that it is a melanosomal protein. In order to investigate its intracellular distribution, we first introduced a VSV-G-epitope at the N terminus of QNR-71 (Fig. 1) to overcome the lack of anti-QNR-71 antibodies able to detect the endogenous protein either by immunoprecipitation or by immunofluorescence. When transiently expressed in primary cultures of pigmented quail cells, E8 dissected from the pigmented layer of the retina (cells from RPE), the epitope-tagged QNR-71 was localized in dotted peripheral structures (Fig. 2a) that, unexpectedly, did not overlap with pigmented melanosomes, also referred as stage III and IV melanosomes (Raposo et al., 2001; Hearing, 1999; Seiji et al., 1963), appearing as a thin black rods by phase contrast (Fig. 2b). An identical distribution was seen when the epitope was placed at the C terminus of QNR-71 or when an HA or a GFP tag were used instead of the VSV-G epitope (data not shown). The absence of QNR-71 staining in this type of fully differentiated melanosomes could be due to its exclusion from this compartment, the masking of the epitope or its rapid degradation when it reaches melanosomes. We conducted pulse-chase experiments to test this latter possibility. Fig. 2c shows that the epitope-tagged QNR-71 was synthesized as a 90 kDa precursor, processed in an approx. 110 kDa mature protein. The epitope-tagged QNR-71 was rapidly degraded exhibiting a short half-life (approx. 1 hour) similar to that of its mouse related gene product PMEL17 (Kobayashi et al., 1994). An identical half-life was measured when the VSG-G epitope was located at the extreme C terminus of the protein or when an HA epitope or a GFP-tag (data not shown) were used instead, indicating that QNR-71 has a rapid turnover. Its degradation occurs in endocytic compartments as it can be reduced upon treatment with ammonium chloride, a treatment which induces the swelling of acidic, endocytic organelles (data not shown). Fig. 2d shows that, upon treatment with ammonium chloride for 1 hour, QNR-71 partially localizes at the rim of swollen structures. Pigments granules were also detected in some of these swollen structures (Fig. 2d) indicating that some of the compartments where QNR-71 resides are somehow connected to the final stage IV melanosomes. A likely explanation for these observations is that QNR-71 is transported to melanosomal compartments where it is rapidly degraded.

We then determined the distribution of QNR-71 in non-pigmented human HEk cells. When transiently expressed in these cells, QNR-71 also distributed to dotted structures. The localization of QNR-71 was then compared with that of several known endocytic markers. First, QNR-71 was consistently detected in EEA1-positive compartments (Mu et al., 1995), indicating that some QNR-71 molecules were present in early endocytic compartments (data not shown). Fig. 2d shows that, upon treatment with ammonium chloride for 1 hour, QNR-71 partially localizes at the rim of swollen structures. Pigments granules were also detected in some of these swollen structures (Fig. 2d) indicating that some of the compartments where QNR-71 resides are somehow connected to the final stage IV melanosomes. A likely explanation for these observations is that QNR-71 is transported to melanosomal compartments where it is rapidly degraded.

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**Schematic representation of the QNR-71 gene product showing the predicted signal sequence (SS), lumenal, transmembrane (TMD) and cytoplasmic domains. The number of amino acids in each domain is indicated. The potential sorting signals in the predicted cytoplasmic domain are shown in bold. The VSV-G epitope is located at the C-terminus of the gene product immediately downstream of the signal sequence.** The gpl-QNR-71 chimeric protein is composed of the lumenal and transmembrane domains of the VZV envelope glycoprotein gpl fused to a part of the QNR-71 tail (amino acids 537 to 556) containing a di-leucine motif.
Fig. 2. Localization and half life of QNR-71 in cells from the quail retinal pigmented epithelium. VSV-G-tagged QNR-71 was transiently expressed in quail cells dissociated from the retinal pigment epithelium at E8. The cells were then fixed and processed for immunofluorescence using the monoclonal P5D4 anti-VSV-G antibody (a,d). (b) The phase contrast image of a, where pigment granules appear as black rod structures. In (c), pigmented quail or HeLa cells were grown on 24-well dishes and transfected with DNAs encoding VSV-G-epitope tagged QNR-71 as indicated in Materials and Methods. The cells were then pulse labeled for 30 minutes with $[^{35}S]$methionine/cysteine and chased for the indicated period of time. QNR-71 was immunoprecipitated with the anti-VSV-G antibody, and analyzed by SDS-PAGE. The position of the precursor (P) and mature (M) forms of the immunoprecipitated proteins are indicated. (d) Cells were first treated with 10 mM NH4Cl for 1 hour before fixation. (d) is the superimposition of phase contrast and fluorescence images. Some of the large vacuoles contain both QNR-71 and melanin pigments.

Fig. 3. Localization of QNR-71 in HeLa cells. HeLa cells were transiently transfected with plasmids encoding the VSV-G tagged QNR-71 and were fixed 48 hours after transfection. They were then processed for indirect immunofluorescence and stained using a polyclonal (a,c) or the monoclonal (e) anti-VSV-G antibody together with the monoclonal anti-EEA1 antibody (b), the monoclonal anti-LampI antibody (d), or a polyclonal anti-Man 6-P/IGF II receptor antibody (f). QNR-71 was detected using FITC-conjugated secondary antibody, while EEA1, Lamp I and Man 6-P/IGF II receptor were decorated using Texas Red-coupled secondary antibodies. Overlaid images are shown on the right.
be more stable in HeLa cells than in pigmented quail cells (Fig. 2c). We also observed a partial co-localization with the Man-6-P/IGF II receptor (Fig. 3e,f), which is mostly present in the trans-Golgi network (TGN) in this cell type (S. Waguri and B.H., unpublished). Treatment with 50 \( \mu \text{g/ml} \) of cycloheximide for 1 hour before fixation led to an almost complete disappearance of QNR-71 from the Man-6-P/IGF II receptor-positive structures (data not shown), indicating that the TGN staining observed in untreated Hela cells most likely represents the newly synthesized QNR-71 en route to endosomes. Thus, when ectopically expressed in non-pigmented cells, QNR-71 behaves in a similar manner to tyrosinase, the major melanogenic enzyme that is located in endocytic organelles (Calvo et al., 1999; Simmen et al., 1999).

**QNR-71 transport and sorting signals**

QNR-71 contains several potential sorting signals similar to those found in proteins transported to endosomal compartments (Fig. 1; Kirchhausen et al., 1997; Le Borgne and Hoflack, 1998b; Mellman, 1996; Sandoval, 1994). Its cytoplasmic tail contains a putative tyrosine-based motif (\( Y^{514}KPI^{517} \)). There is also a di-leucine-based sorting motif with a negatively charged residue in position -4 from the first leucine (\( E^{547}RNPLL^{552} \)). A similar motif is present in other melanosomal proteins such as tyrosinase (Calvo et al., 1999), the major melanogenic enzyme, and TRP-1 (tyrosinase related protein-1) (Jimenez et al., 1991), PMEL17 (Kwon et al., 1991) and NMB (Weterman et al., 1995), and appears to be evolutionary conserved (see Fig. 8; Calvo et al., 1999).

In order to evaluate the relative contribution of these motifs in QNR-71 targeting, we introduced point mutations to alter the tyrosine- (mutant Y514A) or the di-leucine- (L551G, and L552G mutants) based sorting motif (Fig. 1). These different mutants were transiently expressed in pigmented quail cells and in non-pigmented HeLa cells, and their distribution was then examined by immunofluorescence. As shown in Fig. 4, substitution of the tyrosine residue by an alanine did not modify the distribution of QNR-71 in both cell types (Fig. 4e,f). Co-localization studies indicate that the bulk of this mutant is mostly localized in endosomal structures (data not shown). This indicates that this tyrosine-based motif does not substantially contribute to the targeting of QNR-71. By contrast, the point mutation of the leucine552 residue resulted in a significant mislocalization of the mutant proteins to the cell surface in both cell types (Fig. 4c,d). Similar results were also obtained after substitution of the leucine551 by a glycine residue (data not shown). Although these mutants accumulated at the plasma membrane, anti-VSV-G-antibody uptake experiments indicated that they can be internalized (data not shown). Part of the L551G and L552G mutants were also detected in perinuclear compartments in both cell types. As these staining were abolished by cycloheximide treatment, they most probably represent the newly synthesized QNR-71 in the Golgi apparatus en route to the cell surface (not shown). To directly demonstrate that the di-leucine-based sorting signal

**Fig. 4.** Localization of wild-type and mutant QNR-71. The wild-type QNR-71 (a,b), and L552G (c,d) and Y514A (e,f) mutated versions of VSV-G epitope-tagged QNR-71 were transiently transfected in HeLa cells (a,c,e) or pigmented RPE quail cells (b,d,f). Two days after transfection, cells were fixed and processed for immunofluorescence to detect the QNR-71 using the monoclonal anti-VSV-G antibody.

**Fig. 5.** The di-leucine-based sorting signal of QNR-71 is necessary and sufficient for its targeting. The gpI-QNR-71 chimera was transiently expressed together with the wild-type VSV-G-QNR-71 in HeLa cells (a,b) or alone (c). Two days after transfection, cells were fixed, processed for immunofluorescence and stained using a polyclonal anti-gpI antibody (a,c), together with the monoclonal anti-VSV-G antibody (b), or the monoclonal anti-EEA1 antibody (d).
was necessary and sufficient to account for QNR-71 steady state distribution, we fused it to the luminal and transmembrane domains of the reporter glycoprotein gpI (Fig. 1) which are devoid of trafficking information (Alconada et al., 1996). Fig. 5 shows that the chimeric protein co-localizes with the VSV-G-QNR-71 WT construct (Fig. 5a,b). Furthermore, the gpI-QNR-71 chimera also partially co-localized with EEA1 (Fig. 5c,d), LampI and MPRs (data not shown) to a similar extent as the VSV-G-QNR-71 WT construct. Together, these results indicate that the intracellular targeting of QNR-71 to endosomal/lysosomal compartments of pigmented and non-pigmented cells relies mostly on the presence of the di-leucine-based motif.

**AP-3 distribution and expression of QNR-71**

We and others have previously reported that recruitment of AP-1 or AP-3 can be enhanced upon overexpression of selected transmembrane proteins (Alconada et al., 1996; Dittié et al., 1997; Le Borgne et al., 1993; Le Borgne et al., 1998; Salamero et al., 1996; Teuchert et al., 1999). Therefore, QNR-71 was overexpressed in HeLa cells using the T7-RNA polymerase recombinant vaccinia virus as previously reported (Le Borgne et al., 1998). The cells were then fixed, processed for indirect immunofluorescence using either a monoclonal (Fig. 6c) or polyclonal (Fig. 6a) anti-VSV-G antibody to detect the AP-1 complex (Fig. 6b) or a polyclonal anti-δ-adaptin to detect the AP-3 complex (Fig. 6d). As previously reported for lysosomal glycoproteins, overexpression of QNR-71 did not lead to any detectable effect on AP-1 distribution (Fig. 6b). However, overexpression of QNR-71 led to a significant increase in AP-3 staining onto membranes both in the perinuclear region and on the peripheral punctuate structures (Fig. 6d). Some of these peripheral

**Fig. 6.** AP-3 dependent transport of QNR-71. HeLa cells were infected with a recombinant T7 RNA polymerase vaccinia virus and transfected with a plasmid encoding VSV-G-tagged QNR-71. After 3 hours of expression, the cells were fixed and labeled with a polyclonal (a) or monoclonal (c) anti VSV-G antibody and the 109/3 monoclonal anti-γ-adaptin (b) or the polyclonal anti-δ-adaptin (d) antibody to detect AP-1 and AP-3 complexes, respectively. QNR-71 was detected using FITC-conjugated secondary antibodies. γ- and δ-adaptin were decorated with Texas Red-coupled secondary antibodies. Under these experimental conditions, the bulk of the expressed protein is detected in the perinuclear area and has not yet reached the endosomes. Non-transfected cells are indicated with an asterisk.

**Fig. 7.** AP-3 recruitment and expression of QNR-71 mutants. HeLa cells were infected and transfected as indicated in the legend of Fig. 6 with plasmids encoding the L551G (a,b) or Y514A (c,d) mutated forms of VSV-G-tagged QNR-71. After 3 hours of expression, the cells were fixed and processed for indirect immunofluorescence and labeled with the P5D4 monoclonal anti VSV-G antibody followed by a FITC-conjugated donkey anti-mouse antibody (a,c). AP-3 was detected using the polyclonal antibody against the δ-subunit followed by a Texas Red-conjugated donkey anti-rabbit antibody (b,d). Non-transfected cells are labeled with an asterisk.
structures could be decorated with antibodies against the EEA1 (data not shown). The expression of QNR-71 induces an approx. twofold increase in the amount of endogenous AP-3 associated with membranes (see Fig. 8). This AP-3 recruitment was, as expected, totally sensitive to brefeldin A (data not shown). We also overexpressed QNR-71 mutated on its tyrosine-based or on its di-leucine-based motifs. Pulse-chase experiments indicated that these different mutants were expressed at similar levels as the wild-type protein (data not shown). While QNR-71 with a mutated tyrosine-based motif was still able to promote AP-3 recruitment onto membranes as efficiently as the wild-type protein, QNR-71 mutated on leucine 551 (Fig. 7) or leucine 552 (not shown) failed to trigger AP-3 recruitment. A similar increase in AP-3 bound to membranes was also observed upon expression of the gpl-QNR-71 (data not shown), indicating that the di-leucine-based sorting signal of QNR-71 is necessary and sufficient to promote AP-3 recruitment.

Inhibition of AP-3 synthesis and QNR 71 intracellular trafficking

The results described above based on overexpression suggest that QNR-71, like the Lamps and Limps (Dell’Angelica et al., 1999; Le Borgne et al., 1998), follows an AP-3 dependent pathway for its delivery into endosomal/ premelanosomal compartments. This interpretation would be consistent with the fact that expression of QNR-71 results in a measurable mis-sorting of endogenous Lamp1, as seen by antibody uptake experiments (Fig. 9a,b,d,e), indicating that QNR-71 compete for the intracellular targeting machinery required for the lysosomal delivery of Lamp1. Furthermore, we have previously shown that inhibition of μ3A synthesis by antisense oligonucleotides causes a rerouting of endogenous Lamp1 towards the cell surface. If QNR-71 follows an AP-3 dependent pathway, it could therefore be anticipated that a decrease in the amount of functional AP-3 should affect its intracellular targeting. As expected, endogenous Lamp1 does not travel significantly via the cell surface of untransfected cells that are treated (or not) with sense-oligonucleotide, as judged by the inability to internalize exogenously added anti-Lamp1 antibodies (Fig. 9a,b,d,e). In contrast, inhibition of AP-3 synthesis results in a partial misrouting of Lamp1 to the cell surface in these cells (Fig. 9f). Cells expressing QNR-71 exhibit an even higher uptake of anti-Lamp1 antibody when treated with antisense oligonucleotides (Fig. 9f). Anti VSV-G antibody experiments indicate that QNR-71 is partially transiting via the cell surface in every situation. Although we cannot ruled out the possibility that this transport of QNR-71 to the cell surface could be an indirect effect of its ectopic expression, it is interesting to note that endogenous PMEL17 also transits to the cell surface in MNT-1 cells (Raposo et al., 2001). Thus, the cytoplasmic tail of QNR-71 probably contains more sorting information than that of Lamp1. Collectively, these results strongly argue that QNR-71, like the lysosomal glycoprotein Lamp1, makes use of the AP-3 dependent pathway.

DISCUSSION

We report that QNR-71 resides in endosomal/lysosomal compartments of HeLa cells. This melanosomal protein is also detected in melanosomes of pigmented quail neuroretina cells. We also report that, in both pigmented quail cells and non-pigmented HeLa cells, a di-leucine-based sorting signal is necessary and sufficient to allow its intracellular transport, which involves the AP-3 complex.

Intracellular distribution of QNR-71

QNR-71 was originally identified as a quail gene specifically expressed in the pigmented layer of the retina and in the epidermis, as well as during trans-differentiation of quail neuroretina cells after v-Myc transformation (Turque et al., 1996). However, phenotypic pigmentation is not a prerequisite for QNR-71 expression in the pigmented retina, as the corresponding transcripts are detected at E3.5, before pigmentation occurs (Turque et al., 1996). QNR-71 distributes in endosomal compartments, from early to late endocytic structures, in non-pigmented HeLa cells. These results are in good agreement with recent studies showing that tyrosinase exhibits a similar distribution in HeLa cells (Calvo et al., 1999) or MDCK cells (Simmen et al., 1999). While QNR-71 was detected in lysosomes of HeLa cells, the protein was not detected in mature stage IV melanosomes in pigmented cells under normal conditions. As shown using ammonium chloride treatment, our results indicate that QNR-71 partially reaches this compartment where it is possibly rapidly degraded. A similar situation has been reported for PMEL17 in melan-A cells (Kobayashi et al., 1994). It has been proposed that the cytoplasmic domain of PMEL17 is rapidly processed upon arrival in melanosomes (t1/2 is approx. 1-2 hours), while the luminal part of the protein would remain intact. The lysosomal acid phosphatase has also been shown to be processed on its cytoplasmic domain after transport to endosomal compartments (Gottschalk et al., 1989). We have also tested this possibility for QNR-71 by expressing the protein containing an epitope either at the N-terminal or C-terminal part. In both pigmented cells or HeLa cells, no significant difference was observed in the stability of the different
constructs, suggesting that the entire protein is processed. As the function of QNR-71, like that of PMEL17, is unknown, it is possible that they exert a different function than simply being a matrix melanosomal protein. A possibility is rather that QNR-71 resides in unpigmented stage I and II melanosomes, as initially proposed for PMEL17 (Kobayashi et al., 1994; Lee et al., 1996). Interestingly, the gene product related to QNR-71, PMEL17 distributes in premelanosomes of human melanocytes and is almost excluded from stage IV melanosomes at the electron microscopy level (Raposo et al., 2001). Therefore, it is tempting to speculate that QNR-71 may localize into pre-melanosomal compartments.

### Sorting signals in QNR-71 cytoplasmic domain

Our mutational analysis indicates that a di-leucine-based motif present in the cytoplasmic domain of QNR-71 is sufficient to mediate its intracellular transport. A similar motif is found in the cytoplasmic domains of transmembrane proteins highly related to QNR-71, such as the human NMB (Weterman et al., 1995) or PMEL17 (Kwon et al., 1991; see Fig. 10). It can be predicted that these motifs also determine their melanosomal targeting. Such a motif is not only found in other melanosomal proteins such as tyrosinase (Calvo et al., 1999; Kwon et al., 1987, Simmen et al., 1999) or TRP-1 (Jimenez et al., 1991; Vijayasarakhi et al., 1995) but also in lysosomal (LimpII: Ogata and Fukuda, 1994; Sandoval et al., 1994) or vacuolar (Vam3p, Alkaline phosphatase; Darsow et al., 1998) transmembrane proteins. Mutation of this di-leucine motif results in the misrouting of QNR-71 to the cell surface. A similar situation is probably found for PMEL17 (Kwon et al., 1995; Martinez-Esparza et al., 1999). While the function of PMEL17, as well as that of QNR-71 in melanogenesis remains unknown, the silver mutation results in the loss of functional melanocytes in hair follicles (Kwon et al., 1995). Thus, the silver mutation is likely to cause toxic effects to melanocytes analogous to those caused by the phenotypically similar B₄ mutation at the Brown locus (TRP-1; Bennett et al., 1990). Although the reason for this effect is not clear, it is possible that the mutated silver protein could be recognized as a cell surface antigen by cytotoxic T-lymphocytes. Disorders such as vitiligo are also thought to result from the unscheduled destruction of melanocytes by immune mechanisms (Bakker et al., 1994; Kawakami et al., 1994). Thus, a default in trafficking rather than a default in the proper function of PMEL17 might result in melanocyte cell death.

Like QNR-71, several melanosomal proteins contain a tyrosine-based motif located upstream or downstream of the di-leucine-based motif (Fig. 10). As shown here for QNR-71, this determinant is not required for their endosomal targeting. One exception is TRP-2, which lacks the consensus ExxPLL but contains a GYT/APLM sequence. A tyrosine-based signal preceded by a glycine residue has been shown to mediate the lysosomal targeting of LampI (Guarnieri et al., 1993; Harter and Mellman, 1992; Williams and Fukuda, 1990). Thus, TRP-2 and LampI could potentially share common sorting mechanisms, probably by interacting with AP-3 whose μ subunit interacts with tyrosine-based sorting signals (Ohno et al., 1998).

### AP-3-dependent transport of QNR-71

Our study suggests that QNR-71 is transported to endosomal compartments by an AP-3-dependent mechanism. At the microscopic level, overexpression of QNR-71 appears to promote the recruitment of AP-3 onto both perinuclear membranes and EEA1-positive early endosomal compartments. However, no detectable increase of bound AP-3 could be measured by western blotting on purified membranes of cells expressing QNR-71 (data not shown). First, this may be due to the presence of little amounts of AP-3 associated with membranes at steady state or the partial dissociation of AP-3 from membranes during sample preparations. Another possibility is that QNR-71 may only

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**Fig. 9.** AP-3 dependent transport of QNR-71. HeLa cells transfected with the wild-type VSV-G-QNR-71 construct were grown on glass coverslips. They were either left untreated (a,d) or treated with sense (b,e) or antisense (c,f) oligonucleotides. After 48 hours, antibodies directed against Lamp-1 (d-f) and VSV-G (a-c) were added to cell culture medium and allowed to be internalized for 4 hours at 37°C. After extensive washes, the cells were fixed and the internalized antibodies were detected using fluorescently labeled secondary antibodies. Asterisks indicate untransfected cells.
affect the steady-state distribution of AP-3 that now relocates onto QNR-71-positive structures that are better detected at the microscopic level. In any case, the rerouting of endogenous Lamp1 to the cell surface upon QNR-71 expression indicates that QNR-71 and Lamp1 compete for the same AP-3-dependent machinery. Furthermore, the inhibition of AP-3 synthesis combined with the expression of QNR-71 further increases the misrouting of both proteins via the cell surface.

The recruitment/relocalization of AP-3 depends on an intact di-leucine-based motif present in the ExxPLL sequence. No effect was observed for the related AP-1 adaptor complex. Our results from this in vivo study agree well with in vitro binding experiments showing that the cytoplasmic domain of tyrosinase is able to interact with AP-3 using a di-leucine based motif (Höning et al., 1998). They are also in good agreement with the required sequence present in the cytoplasmic domain of the alkaline phosphatase and in Vam3p for the AP-3-dependent vacuolar protein sorting in yeast (Darsow et al., 1998). As AP-3 is also involved in the intracellular transport of lysosomal glycoproteins (Dell’Angelica et al., 1999; Le Borgne et al., 1998), this adaptor complex may function in the biogenesis of both lysosomes and melanosomes. Indeed, although the major phenotypically visible defects of mutations in the different subunits of AP-3 are pigmentation defects in the eye and other tissues of Drosophila (Kretzschmar et al., 2000; Mullins et al., 1999; Ooi et al., 1997; Simpson et al., 1997), coat color

Fig. 10. Cytoplasmic domains of melanosomal, lysosomal and vacuolar transmembrane glycoproteins. Sequence alignment of the cytoplasmic domain of integral membrane proteins targeted to lysosomes/vacuole or melanosomes. Boxed are the di-leucine- and tyrosine-based sorting signals from the indicated proteins. The indicated numbers reflect the number of amino acids between two domains or two signals. The most conserved residues are indicated in bold. Sequences were obtained from GenBank.

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The AP-3 pathway is evolutionary conserved from the yeast Saccharomyces cerevisiae (Cowles et al., 1997; Stepp et al., 1997; Vowels and Payne, 1998) to high eukaryotic cells (Dell’Angelica et al., 1999; Feng et al., 1999; Kantheti et al., 1998; Le Borgne et al., 1998; Zhen et al., 1999). A basal level of expression of the different AP-3 subunits is likely to be sufficient for lysosomal targeting. However, melanogenesis requires the expression and targeting of additional sets of enzymes involved in this process, including tyrosinase, TRP-1 and TRP-2, and also several transporters such as tyrosine and zinc transporters, leading to the accumulation of melanin inside newly formed melanosomes. Thus, it is possible that AP-3 could be upregulated during the process of melanogenesis. We have compared the expression levels of the μ- and ε3a,b subunits of AP-3, in non-pigmented or pigmented quail neuroretina cells at different stages of pigmentation. We did not observe any significant differences in the level of expression of these two AP-3 subunits (not shown). As the formation of pigments in melanoblasts requires around 14 days in culture, it would appear that, at least in this cell system the transport machinery is expressed before the appearance of fully mature melanosomes. Furthermore, some lysosomal and melanosomal glycoproteins follow an AP-3-dependent pathway for their intracellular delivery in endocytic structures. It could be envisaged that this part of the transport machinery is common to both pathways. This would explain why there is no need to increase the production of AP-3 complexes during melanogenesis. Later in the endocytic pathway, the two classes of glycoproteins would be diverted away by different means to allow the formation of two distinct organelles. Maybe only this second step, for example, a maturation process that can be AP-3 independent, would be melanocyte specific. A candidate gene for such a process in mice is cappucino because defects in this gene cause a Hermanolsky-Pudlak syndrome in which AP-3 is not involved (Gwynn et al., 2000). Other genes that could function downstream of AP-3 such as lyst, pallid (a gene coding a syntaxin 13-interacting protein; Huang et al., 1999), or gunmetal, a gene coding for a Rab geranyl-geranyl transferase, could also participate in melanosome targeting (Selaturi, 2000; Swank et al., 2000).

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