The tyrosinase tail mediates sorting to the lysosomal compartment in MDCK cells via a di-leucine and a tyrosine-based signal

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SUMMARY

Tyrosinase is a type I membrane protein found in melanosomes, which are lysosomal-like organelles and specific for pigment cells. A mutation of mouse tyrosinase, platinum (ε°), leads to truncation of tyrosinase’s cytosolic tail, and results in misrouting to the cell periphery. In this study, we expressed chimeras of wild-type and mutant cytosolic tails of mouse tyrosinase fused to rat lysosome-associated membrane protein-1 luminal and transmembrane domain to study sorting of tyrosinase in Madin-Darby canine kidney cells. The study shows that the mouse tyrosinase cytosolic tail is necessary and sufficient to mediate sorting of a heterologous type I membrane protein to compartments of the lysosomal lineage. Whereas deletions of 7 or 10 C-terminal amino acids of the tail still result in sorting to lysosomes, a deletion mutant corresponding to platinum (ε°) tail fails to sort correctly and corroborates the in situ findings in ε° homozygous mutant mice. Correct sorting of tyrosinase-lysosome-associated membrane protein-1 chimeras is mediated by the interplay of a di-leucine signal and a tyrosine motif of the Y-X-X-Ø type.

Key words: Tyrosinase, Intracellular sorting, Melanosome, Lysosome, c-Locus, AP-3

INTRODUCTION

Melanosomes are thought to represent specialized lysosomes (reviewed by Orlow, 1995). They are the site of biosynthesis of the pigment melanin which is a tyrosine-based biopolymer. The close relationship between melanosomes and lysosomes is illustrated by the autosomal recessive disorder Chediak-Higashi syndrome (CHS) in humans and the corresponding mouse mutation beige, which both result in oculocutaneous albinism, and affect equally melanosomes and lysosomes (Barak and Nir, 1987; Barbosa et al., 1996; Burkhardt et al., 1993). CHS stems from a defect in the lysosomal traffic regulator protein (LYST) which has been postulated to play a role in microtubule-dependent transport into late endocytic compartments (Faigle et al., 1998). Similarly, Hermansky-Pudlak syndrome, a recessive disease found in humans and in mice, is characterized by abnormalities in lysosomes, melanosomes and platelets (Swank et al., 1998). Like lysosomes, melanosomes have a very low intraorganelar pH of 3-5 (Bhatnagar et al., 1993). Lysosomal markers LAMP-1 and -2 (lysosome-associated membrane protein) are also found in melanosomes (Orlow et al., 1992; Zhou et al., 1993), as are lysosomal hydrolases (Diment et al., 1995).

Melanosomes differ from lysosomes by the presence of enzymes involved in pigment synthesis, as for example tyrosinase (EC 1.14.18.1), a protein with a molecular mass of about 68 kDa when glycosylated (Hearing and Tsukamoto, 1991). Tyrosinase is regarded as the key enzyme in melanin synthesis, and catalyzes the first two reactions, the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPAquinone (del Marmol and Beermann, 1996; Hearing and Tsukamoto, 1991). A further cascade of reactions then leads to the melanin biopolymer (del Marmol and Beermann, 1996; Hearing and Tsukamoto, 1991). In mouse, tyrosinase is encoded at the c (albino) locus, and mutations at this locus have been known for decades (Jackson, 1994). A nonfunctional tyrosinase enzyme, found for example in the original albino mutation (c), leads to complete absence of pigmentation in skin, hair and eyes (Beermann et al., 1990; Jackson, 1994; Ruppert et al., 1988). In addition to tyrosinase, melanosomes contain two other proteins of the tyrosinase-related protein (TRP) family, TRP-1 and TRP-2, which are both implicated in eumelanin synthesis (del Marmol and Beermann, 1996; Jackson, 1994). Intracellular trafficking has been studied for TRP-1. Using transfections of fibroblasts and B16 melanoma cells, it has been shown that a C-terminal di-leucine motif is required for correct sorting to lysosomes/melanosomes (Vijayasaradhi et al., 1995).

The fact that the Drosophila garnet mutation, which shows diminished eye coloration, is linked to mutations in the delta subunit of the adaptor protein AP-3 has stimulated interest in
the trafficking of melanosomal proteins (Odorizzi et al., 1998; Ooi et al., 1997; Simpson et al., 1997). A recent study (Höning et al., 1998) has examined the binding of the cystolic tail of tyrosinase to AP-3 by surface plasmon resonance (SPR). It was found that a di-leucine signal in the cystolic tail of tyrosinase interacts with AP-3 (Höning et al., 1998). Furthermore, AP-3 has been shown to associate with clathrin (Dell’Angelica et al., 1998) and to bind to cystolic tails of membrane proteins (Odorizzi et al., 1998).

The analysis of pigment cells from mice homozygous for the mouse c locus mutation platinum (c*) (Beermann et al., 1995) indicated, that platinum tyrosinase is not found in melanosomes, but is rather misrouted to the cell surface. Molecular analysis revealed that the cystolic tail is lacking, due to a point mutation introducing a termination codon (Beermann et al., 1995). Based on this result, we further analyzed potential in vivo sorting signals in the tyrosinase tail, by using transfections of rat LAMP-1/mouse tyrosinase chimeric constructs. Our results show: (1) failure of a platinum (c*) tyrosinase mutant tail to mediate sorting; (2) importance of a di-leucine signal in correct sorting; and (3) the involvement of a tyrosine-based signal of the Y-X-X-O type.

**MATERIALS AND METHODS**

**Construction of tail mutants**

LAMP-1/tyrosinase chimeric constructs were based on a modified rat LAMP-1, cloned in the expression vector pcB6, with a unique AflII site introduced after the transmembrane domain (Höning and Hunziker, 1995). Wild-type (LLT) and platinum (c*) LLTAS06) tyrosinase tails were amplified by PCR using mouse tyrosinase cDNA (wild type or platinum; Beermann et al., 1995) as template, thereby creating an AflII site in the presumptive transmembrane region of tyrosinase (primer 1: 5′-gctgatgttgtgcccttcctt-3′, +1569 to +1589; primer 2: 5′-ctagatgttgtgcccttcctt-3′, +1835 to +1816; Müller et al., 1988; Schmidt and Beermann, 1994). The resulting PCR fragment was subcloned as a AflII/SpeI (SpeI +1760) fragment into the AflII/XbaI-cut vector. Construction of deletion mutants LLT526, LLT525, LLT516, LLT511 was essentially as follows: PCR fragments with the desired mutation and carrying an XbaI site in the non-coding region were cut AflII/XbaI and ligated to replace the tyrosinase wild type tail in the LLT construct. The following 3′ primers were designed: LLT526: 5′-gtctTCAGAcgttgctgcttcctt-3′, LLT525: 5′-gcaarTCAGAcgttgctgcttcctt-3′, LLT516: 5′-tctgTCAGAcgttgctgcttcctt-3′, LLT511: 5′-gtctTCAGAgtgctgcccttcctt-3′.

Point mutants LLT Y524A and LLT LL517,518AA were obtained by two successive PCR reactions using sense and antisense mutant primers (Y524A: 5′-agatgtgatgtgcttcctt-3′ and 5′-gctgatgttgtgcccttcctt-3′, LL517, 518AA: 5′-tctgtgcccttcctt-3′ and 5′-ctagatgttgtgcccttcctt-3′) in combination with 5′ and 3′ primers covering the complete wild-type tail. The first two independent PCR reactions resulted in two shorter products, which overlap at the mutant oligonucleotide. They were subjected to a second PCR reaction, using primers at the 5′ and 3′ end of the tail (as used for creation of the LLT construct). The AflII/SpeI-cut PCR product was cloned into AflII/XbaI-cut vector. The constructs containing replacements of the 5 C-terminal amino acids by alanines were created as described above for deletion mutants, using LLT wild type or LLT LL517, 518AA as templates (LLT 529-533A and LLT LL517, 518AA: 5′-ctgTCAGAcgttgctgcttcctt-3′, LLT Y524A: 5′-agatgtgatgtgcttcctt-3′, LLT 529-533A: 5′-gctgatgttgtgcccttcctt-3′). PCR amplification and cloning of all constructs was corroborated by sequence analysis.

**Stable transfections**

Constructs were transfected into MDCK clone II cells, grown in DMEM supplemented with 10% FCS, using the calcium phosphate method (Hunziker and Mellman, 1989). Stable transfectants were selected in G418-containing medium and picked. Positive clones were identified by immunofluorescence microscopy with polyclonal rabbit anti-lgp120 antibody (Höning and Hunziker, 1995).

**Transient transfections of MDCK cells**

One day prior to transfection, cells were plated on a 30 mm dish containing 3 or 4 round coverslips (12 mm diameter) to a density of 20-30%. A mixture of 6 μg DNA (construct) and 12 μl 2 M CaCl2 (in 100 μl H2O) was added to 100 μl of a 2× concentrated Hepes buffer (280 mM NaCl, 50 mM Hepes, 1.5 mM Na2HPO4 × 2H2O, pH 7.1-7.3), mixed and added dropwise to the cells (grown in DMEM/10% FCS). After 24 hours incubation, medium was replaced by DMEM/10% FCS containing 10 mM butyrate to increase expression from the CMV promoter. The following day, coverslips were processed for immunofluorescence. We did not expect that butyrate alters steady state distribution of tyrosinase. However, butyrate might induce sorting via the surface for proteins which are normally targeted directly to lysosomes (Höning and Hunziker, 1995).

**Immunofluorescence and endocytosis assays**

For detection of LAMP-1/tyrosinase fusion constructs a polyclonal rabbit antibody was used (Mingus; see Höning and Hunziker, 1995). Endogenous canine LAMP-2 was detected using monoclonal mouse antibody AC17 (Nabi et al., 1991). Endogenous mouse tyrosinase was detected by polyclonal rabbit antibody pEP7 (Jiménez et al., 1991).

Stable transfectants were analyzed in the absence of butyrate. Cells grown on glass coverslips were fixed for 2 minutes in methanol at −20°C. Nonspecific binding sites were blocked with 10% goat serum in PBS for 30 minutes and the cells were incubated with a 1:100 dilution of polyclonal or monoclonal antibody (30 minutes in 10% goat serum in PBS). Detection of the primary antibodies was done using a 1:100 dilution of the corresponding secondary antibodies (10% goat serum in PBS). Coverslips were mounted in Moviol and viewed on a Zeiss Axiophot microscope using a x63 or x100 Apachromat oil immersion lens. Pictures were acquired using a Color Cool View camera (Photonic Science) and Image Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA) and processed with Photoshop 4.01 software (Adobe Systems, Inc.).

For endocytosis assays, cells were grown on glass coverslips. Endocytosis of LAMP-1/tyrosinase fusion constructs was assayed by immunofluorescence after internalization of polyclonal or monoclonal antibody for 1 hour at 37°C (Höning and Hunziker, 1995). Colocalization with lysosomal marker (LAMP-2) was done by coincubating immunofluorescence preparations with a primary mouse monoclonal antibody against endogenous LAMP-2 and the polyclonal rabbit antibody against the rat LAMP-1 luminal domain. No crossreactivity was observed.

**RESULTS**

**Tyrosinase/LAMP-1 chimeras are correctly targeted to lysosomes in MDCK cells**

To study intracellular sorting of mouse tyrosinase, we chose transfections of tyrosinase constructs in the canine kidney cell line MDCK, a well-established model for intracellular trafficking. Full-length mouse tyrosinase, when transiently expressed in MDCK cells (Fig. 1D), is localized to lysosomes (Fig. 1E,F), when compared to the immunofluorescence expression patterns of an endogenous lysosomal protein, LAMP-2 (Fig. 1). The commonly used antiserum against
Sorting of tyrosinase tail

Mouse tyrosinase (αPEP7) is directed against the C terminus (Jiménez et al., 1991). No antibody has been described, to our knowledge, which recognizes the mouse tyrosinase N terminus or luminal domain in immunofluorescence. In preliminary experiments, we therefore tried to circumvent this problem by using a FLAG-tagged tyrosinase construct. Due to unknown reasons, the tagged protein was retained in the endoplasmic reticulum (ER) (data not shown). We concluded that the missorting of tagged tyrosinase was caused by the introduction of the FLAG-tag and not due to aberrant sorting in MDCK cells.

We then fused the cytosolic tail of tyrosinase to the luminal and transmembrane domain of the lysosomal membrane glycoprotein LAMP-1, due to its analogous localization to tyrosinase in both MDCK and pigment cells (see above; Orlow et al., 1992; Zhou et al., 1993). We first analyzed whether this construct results in correct targeting in MDCK cells. Like the transient transfections with full-length tyrosinase, MDCK cells expressing the LAMP-1-tyrosinase chimera (Fig. 1A) show colocalization with canine LAMP-2 (Fig. 1B,C), whereas non-transfected MDCK cells show no staining for LAMP-1 (not shown; Höning and Hunziker, 1995). These data rule out a possible retention of the chimera in the ER, as is the case of the tagged form of tyrosinase in our previous experiment or of tyrosinase in amelanotic melanoma cells (Halaban et al., 1997). We therefore conclude that the LAMP-1-tyrosinase chimera is sorted to the lysosomal compartment in the absence of melanosomes.

The tyrosinase tail encodes a melanosomal/lysosomal sorting determinant based on a di-leucine motif

Having shown that correct sorting of LAMP-1 tyrosinase chimera takes place in MDCK cells, we constructed a series of deletion mutants (Fig. 2A). In the cytosolic tail of tyrosinase, two potential regions for a lysosomal/melanosomal sorting signal are present: (i) the region around tyrosine 524 of mouse tyrosinase (numbering beginning at ATG of tyrosinase), which corresponds to a sorting signal of the type Y -X-X-Ø where Ø stands for any bulky hydrophobic amino acid; (ii) the di-leucine motif at position 517/518. The deletion mutants were designed to determine the importance of these potential motifs for the sorting of tyrosinase. In addition, we also examined the sorting of a chimeric protein containing platinum (cP) tyrosinase tail (LLTΔ506). The c locus mutation platinum (cP) causes extreme hypopigmentation and is characterized by presence of a premature termination codon (Beermann et al., 1995).

Fig. 1. (A-C) Colocalization (C) of endogenous canine LAMP-2 (B) with a transfected wild-type tyrosinase cytosolic tail/LAMP-1 luminal domain and transmembrane domain fusion construct (A) in MDCK cells. (D-F) Colocalization (F) of endogenous canine LAMP-2 (E) with transiently transfected mouse tyrosinase (D) in MDCK cells. Cells were fixed and permeabilized with methanol. Staining was revealed by coincubating the different primary antibodies and binding of the appropriate secondary antibodies. Bar, 13 µm.

Fig. 2. Mutants of mouse tyrosinase tail. LLT corresponds to LAMP-1 luminal and transmembrane domain fused to tyrosinase cytosolic tail. The numbers refer to the mouse tyrosinase amino acid sequence. Deletion and point mutants are listed in A and B, respectively. ∆ indicates deletion of tail sequence up to the respective amino acid. LLTΔ506 is also known as the tail of the platinum (cP) mutant tyrosinase resulting in extreme hypopigmentation in mice (Beermann et al., 1995).

A. Deletion Mutants

| Wildtype LLT | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT Δ526 | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT Δ523 | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT Δ516 | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT Δ511 | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| (cP) LLT Δ506 | LKKKKK |

B. Point Mutants

| LLT Y524 → A | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT L517, 518 → AA | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT S529-S533→A | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT Y524 → A, S529-S533 → A | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
| LLT L517, 518 → AA; S529-S533→A | LKKKKKKKQPQERQP1LMMDYHSYLLYQSHL |
The sorting of these deletion mutants was examined by steady state immunofluorescence and an endocytosis immunofluorescence assay. As expected, wild-type LLT localizes to intracellular lysosomal structures (Fig. 3A) and can be internalized from the cell surface to these compartments (Fig. 3B). The same is true for LLT Δ526 and LLT Δ523 (Fig. 3C-F), even though the tyrosine-based sorting signal is destroyed or deleted. As can be seen for the deletion LLT Δ516 (Fig. 3G,H), the presence of the di-leucine motif is crucial, since this construct is neither localized to intracellular structures, nor able to internalize. LLT Δ511 (not shown) and LLT Δ506 (platinum, □□) both show surface staining and no internalization (Fig. 3I,J). The absence of internalization was confirmed by confocal laser immunofluorescence microscopy after stripping surface-bound antibody with low pH medium (not shown).

![Steady state and endocytosis images](image-url)
The results demonstrated that the platinum tyrosinase cytosolic tail results in misrouting to the cell periphery. The results furthermore suggested, that the di-leucine motif LL 517/518 is necessary for targeting of tyrosinase to compartments of the lysosomal lineage. In addition, wild-type tyrosinase cytosolic tail not only confers localization to the lysosomal/melanosomal compartment, but also enables the LLT fusion construct to be internalized from the surface. Point mutants show requirement of di-leucine motif and Y-X-X-Ø sorting signal for correct localization

According to the results of the deletion mutants, presence of the di-leucine motif is important for sorting to lysosomes in MDCK cells. We therefore mutated this motif (LL 517/518) to two alanines in the context of the wild-type tyrosinase tail (Fig. 2B). When the mutant was examined by steady state and endocytosis immunofluorescence, it showed staining on the surface and no internalization (Fig. 4A,B). Thus, the di-leucine motif is absolutely essential for correct sorting.

It had been reported recently using two-hybrid screening that the medium chain adaptor subunit of AP-3 can interact with tyrosine-based sorting signals (Dell'Angelica et al., 1997). The sequence YSHL (residues 524-527) in the cytosolic tail of tyrosinase is a sequence of the type Y-X-X-Ø. We therefore mutated Y524 to alanine in context of the wild-type tail (Fig. 2B). Again, this mutant construct sorted to the cell periphery, without internalization (Fig. 4C,D). Correct sorting of tyrosinase therefore requires presence of both a di-leucine and a Y-X-X-Ø signal. Apparently, the di-leucine signal can be sufficient for melanosomal targeting in the context of the deletion mutants, whereas the tyrosine-based signal is revealed to be necessary in the context of the whole tail.

Mutation of C-terminal amino acids enables independent recognition of both sorting signals

The results presented so far have shown a discrepancy between certain deletion mutants and the point mutations. Whereas the deletion of the Y-X-X-Ø motif still allows the fusion proteins to be internalized (Fig. 3), mutation of the tyrosine (Y524A) completely abolishes correct sorting. Instead, the fusion protein is targeted to the surface (Fig. 4). The difference between point mutations and deletion mutants is the presence or absence of the C-terminal amino acid residues of the cytosolic tail of tyrosinase. We therefore replaced residues 529-533 by alanines to establish a possible negative interaction with the tyrosine-based sorting signal (Fig. 2B).

Indeed, this mutation restored intracellular localization of LLT fusion proteins (Fig. 5A,C,E), even though not completely in construct LLT LL517, 518 AA; 529-533AA (Fig. 5E,F). In addition to the intracellular staining, a considerable amount of protein is found on the surface when permeabilization is omitted in the standard protocol (not shown). Construct LLT 529-533A shows slightly more protein on the surface in the endocytosis assay (Fig. 5B). Thus, the last 5 C-terminal amino acids may be important for efficient recognition of the two sorting signals.

Localization of constructs to lysosomes following endocytosis or steady state

Point mutants and deletion mutants differed with regard to the importance of the tyrosine-based motif. We therefore examined if this sorting signal is necessary and sufficient for correct endocytosis to lysosomes. Colocalization with endogeneous canine LAMP-2 was analyzed using the monoclonal antibody AC17 (Fig. 6). For all intracellularly localized constructs, colocalization was found under steady state conditions (Fig. 6). For the wild-type fusion construct, colocalization was also found in the endocytosis assay. Mutation of the 5 C-terminal amino acids still allows endocytosis to lysosomes, even though the presence of some residual surface staining indicates that the efficiency of internalization may be affected (Fig. 6D). However, mutation of both the tyrosine and the 5 C-terminal amino acids (LLT Y524A; 529-533AA) seems to affect correct internalization to lysosomes (Fig. 6F).
Fig. 5. Steady state distribution and endocytosis immunofluorescence assay in mutants of the 5 C-terminal amino acid residues. Cells were treated as described (Fig. 3). Mutation of the 5 C-terminal amino acids to alanines allows a point mutant of tyrosine 524 (Y524A) to be internalized (C,D), in contrast to the same mutation in wild-type context (Fig. 4D). Mutation of the 5 C-terminal amino acids to alanines did not affect the sorting behavior in the wild-type context (A,B). In the context of the mutation of the di-leucine sorting signal endocytosis seems to be restored to a minor extent (E,F). Bar, 18 µm (A,E,F); 10 µm (B,C,D).

Fig. 6. Colocalization of LAMP-1/tyrosinase constructs at steady state and endocytosis conditions. Stably transfected cells were grown on coverslips and treated according to standard immunofluorescence protocols with primary antibodies recognizing tyrosinase and LAMP-2, respectively. DTAF secondary antibody against LAMP-2 and CY3 secondary antibody against transfected LAMP-1/tyrosinase constructs were added. Constructs show overlapping staining for steady state conditions (A,C,E). For endocytosis, this is seen for the LLT wild-type construct (B; this was overexposed to illustrate colocalization), but not seen when tyrosine 524 is mutated in addition to the mutation of the 5 C-terminal amino acids (F). Mutation of the 5 C-terminal amino acids alone allows endocytosis to lysosomes, even though some surface staining is observed (D). Bar, 4 µm (B,C,E,F); 8 µm (A,D).
DISCUSSION

Melanosomes are organelles of the endolysosomal lineage (Orlow, 1995, 1998). In nonmelanocytic cells, when transfected using expression vectors, TRP-1 or tyrosinase localize to granules positive for LAMP-1 and lysosomal hydrolases (Vijayasaradhi et al., 1991; Winder, 1991; Winder et al., 1993). This demonstrates that, in cells lacking melanosomes, the targeting information of melanosomal proteins is necessary and sufficient to sort the proteins to lysosomes. We therefore used MDCK cells to analyze the tail of one of these proteins, tyrosinase, fused to the luminal/transmembrane domain of a well characterized protein, LAMP-1 (Höning and Hunziker, 1995). We were obliged to perform the experiments using chimeric constructs, since the only available antibody, aPEP7, which allows detection of mouse tyrosinase in immunofluorescence, is directed against the C terminus (Jiménez et al., 1991). Previous results had indicated that sorting of tyrosinase to the melanosomal/lysosomal compartment might be governed by the cytosolic tail. A mouse mutant at the c locus, platinum (c<sup>p</sup>), is characterized by a truncation of the last 27 amino acids. The resulting tyrosinase protein still behaves as an integral membrane protein, although it is not found in melanosomes, but is misrouted instead to the cell surface (Beermann et al., 1995).

The results presented here show that at least two sorting determinants are present within the tyrosinase tail. One of them is a di-leucine signal which by itself is sufficient for targeting tyrosinase to its destination. Another is a tyrosine-based sorting signal (Y-H-S-L) of the type Y-X-X-Ø, with Ø being a bulky hydrophobic amino acid. This signal may be important for the final targeting step of tyrosinase to the lysosome if trafficking on an indirect pathway via the cell surface. Although we cannot exclude the possibility that the two motifs are part of a combined signal, similar signals can interact independently with adaptor proteins (Heilker et al., 1996; Höning et al., 1996; Ohno et al., 1995). These two sorting signals are conserved, and found in cytosolic tails of different vertebrate tyrosinases and, similarly, in those of the tyrosinase-related proteins TRP-1 and TRP-2 (Fig. 7). More importantly, the two sorting signals we have identified to be important for lysosomal/melanosomal targeting are the only ones conserved between species. In tyrosinase and TRP-1, both the tyrosine-based sorting signal and the di-leucine signal are always preceded by acidic amino acids possibly playing a role in recognition of the signals. The importance of the C-terminal amino acids is more complex: this stretch of amino acids might interact with the two sorting signals as evidenced by the restored endocytosis ability of the double mutants (Fig. 5). This sequence is not conserved between melanosomal proteins (Fig. 7) and does not fit any sorting motif consensus. Therefore, it is not clear whether this represents a signal on its own, or just a sequence requirement for recognition and exposure of the other two signals.

It has been suggested that the adaptor protein AP-3 has a role in sorting events to the melanosome (Dell’Angelica et al., 1997; Simpson et al., 1997). Recent in vitro results using synthetic peptides and affinity columns demonstrated that the cytosolic tail of mouse tyrosinase is able to interact with the medium chain subunit of AP-3 (µ<sub>3</sub>; Höning et al., 1998). The data from Höning et al. (1998) entirely confirm the requirement of the identified di-leucine motif. They also described a more distal di-leucine motif in the mouse tyrosinase tail (L527/L528) which seemed to be only of minor importance for binding of AP-3. It is not clear yet whether the tyrosine-based sorting signal we have identified extends until this distal di-leucine motif or not.

Mutation of tyrosine in addition to the last C-terminal amino acids (LLT Y524A; 529-533A) seemed not to affect steady state distribution and localization to lysosomes (Fig. 6E). However, following endocytosis, this mutant failed to colocalize with LAMP-2 (Fig. 6F). It is unlikely that this is due to a kinetic difference, since antibody was allowed to internalize for 1 hour. Rather, since AP-3 is present on the endocytic compartment and may play a role in the transfer from early to late endocytic compartments (Ooi et al., 1998), an impaired binding of LLT Y524A; 529-533A to AP-3 may prevent its transfer to LAMP-2 positive compartments in the endocytic route. Nevertheless, a fraction of the construct may still be targeted to the lysosomal system via an AP-3-independent pathway, possibly from the TGN via AP-1-coated vesicles.

Which route does tyrosinase take to reach the melanosome? Our results obtained with chimeric constructs indicate that it can take an indirect route via the surface. They do not exclude
the possibility, however, that a fraction is directly delivered from the TGN to the melanosome. Our immunofluorescence results suggest that the tyrosine-based signal is important for final delivery to the lysosomal compartment in the indirect pathway. A direct pathway may involve recognition by AP-3, which has been shown in yeast (Cowles et al., 1997) to deliver cargo to the vacuole, the yeast equivalent of the lysosome, without passage through the endosome. On the other hand, there is evidence also for an involvement of endosomes in melanosomal targeting: in addition to the garnet gene product, another pigmentation defect in Drosophila, dor (deep orange) has been linked to a lysosomal sorting defect. Surviving dor mutant flies exhibit defects in pigment deposition within the eyes, ocelli, fat body, and malpighian tubules (Lindsay and Zimm, 1992; Tearable, 1991). The dor protein has been identified and shares significant homology with the yeast VPS18 gene product (Shestopal et al., 1997), which is important for sorting to the vacuole on the two routes, involving either passage through endosomes or recognition by AP-3 (Rieder and Emr, 1997).

Recently, it has been proposed (Reaves et al., 1998) that the luminal and the transmembrane domain of LAMP-1, as used in our experiments, also contain lysosomal targeting information. However, these findings do not interfere with our interpretations, since: (i) both tyrosinase and LAMP-1 are targeted to lysosomes in MDCK cells (Fig. 1); and (ii) several mutants show surface staining despite the presence of LAMP-1 transmembrane and luminal domains (Figs 3, 4).

In summary, our data have identified a di-leucine and a tyrosine-based sorting motif within the cytosolic tail of the melanosomal protein tyrosinase. We have characterized these motifs within an epithelial cell type, MDCK, by using a lysosomal protein as a reporter molecule. Nevertheless, we believe that the results reflect the situation of wild-type tyrosinase in normal pigment cells: (i) epithelial pigment cells exist in the retinal pigment epithelium (Zhao et al., 1997); (ii) melanosomes are specialized lysosomes (Orlóy, 1995); (iii) transfected tyrosinase is transported to lysosomes in non-pigment cells (Winder, 1991); (iv) tyrosinase in mice homozygous for the c locus mutant platinum is found at the cell surface (Beermann et al., 1995); (v) a platinum tail reflects failure to sorting (Fig. 3); and (vi) the identified motifs are conserved (Fig. 7; Orlóy, 1998; Vijayasaradhi et al., 1995).

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Sorting of tyrosinase tail


