A Cytoplasmic Sequence in Human Tyrosinase Defines a Second Class of Di-leucine-based Sorting Signals for Late Endosomal and Lysosomal Delivery*

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Distinct cytoplasmic sorting signals target integral membrane proteins to late endosomal compartments, but it is not known whether different signals direct targeting by different pathways. The availability of multiple pathways may permit some cell types to divert proteins to specialized compartments, such as the melanosome of pigmented cells. To address this issue, we characterized sorting determinants of tyrosinase, a tissue-specific resident protein of the melanosome. The cytoplasmic domain of tyrosinase was both necessary and sufficient for internalization and steady state localization to late endosomes and lysosomes in HeLa cells. Mutagenesis of two leucine residues within a conventional di-leucine motif ablated late endosomal localization. However, the properties of this di-leucine-based signal were distinguished from that of CD3γ by overexpression studies; overexpression of the tyrosinase signal, but not the well characterized CD3γ signal, induced a 4-fold enlargement of late endosomes and lysosomes and interfered with endosomal sorting mediated by both tyrosine- and other di-leucine-based signals. These properties suggest that the tyrosinase and CD3γ di-leucine signals are distinctly recognized and sorted by distinct pathways to late endosomes in non-pigmented cells. We speculate that melanocytic cells utilize the second pathway to divert proteins to the melanosome.

The maintenance of morphological and functional integrity of individual compartments of the secretory and endosomal pathways requires proper sorting of integral membrane components. Eukaryotic cells have evolved mechanisms to divert specific integral membrane protein components from several sites into tubular and/or vesicular carriers destined for late endosomes and lysosomes (reviewed in Refs. 1 and 2). Most of these mechanisms require the recognition of specific sorting signals within the cytoplasmic domains of resident proteins by cytosolic factors. It is becoming increasingly clear, however, that there are redundant pathways to late endosomes and lysosomes in many eukaryotic cells. An example of this redundancy is observed in Saccharomyces cerevisiae, in which two apparently independent pathways with distinct effectors converge on the lysosome-like vacuole to deliver different sets of hydrolases and structural components (3, 4). Cargo selection for these two pathways relies on the recognition of distinct cytoplasmic sorting signals (5–8). Evidence also exists in mammalian cells for multiple pathways from the trans-Golgi network to late endosomes (9–13), but sorting signals required for entry into different pathways have not yet been clarified.

What advantage would be imparted to an organism by the evolution of independent pathways to late endosomes and lysosomes? For higher eukaryotes, one potential advantage would be the ability to subvert one pathway or the other in specific cell types to permit the development of unique, tissue-specific lysosome-like organelles. Such a strategy would be similar to that used to establish polarity in epithelial cells. Protein sorting to the basolateral and apical cell surfaces requires sorting signals that are recognized for packaging into distinct vesicular carriers in both polarized and non-polarized cells (14–16), but only in polarized cells are the vesicles targeted to different destinations (reviewed in Ref. 17). Whether comparable mechanisms act in protein sorting to other tissue-specific post-Golgi organelles is not known.

One tissue-specific organelle with the potential for accumulating a subpopulation of proteins diverted from the late endosome is the melanosome, the organelle of melanocytes in which melanin is synthesized (18, 19). Melanosomes resemble secretory lysosomes in that they are highly acidic and often co-fractionate with markers of late endosomes and lysosomes such as lamp1, lamp2, CD63, and acid phosphatase (20–23). Furthermore, genes that control coat color in mice and skin color in humans encode proteins that are involved in lysosomal biogenesis (24, 25). Nevertheless, melanosomes contain a unique cohort of resident membrane proteins and can be distinguished from late endosomes and lysosomes in some pigmented cells (26). Thus, resident proteins of both organelles must be differentially sorted, but the characteristics that distinguish between sorting to late endosomes/lysosomes and to melanosomes have not been established.

Perhaps the best characterized melanosomal resident protein in normal and malignant melanocytes is tyrosinase (reviewed in Ref. 28). Human tyrosinase, the rate-limiting enzyme in melanin biosynthesis, is a melanocyte-specific, type I integral membrane glycoprotein with a large luminal domain, a 26-amino acid transmembrane domain, and a 30-amino acid cytoplasmic domain (Ref. 27; see Fig. 1 for schematic). Impairment of tyrosinase activity in humans results in oculocutaneous albinism (OCA1; Ref. 29). Among the many tyrosinase gene

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mutations that cause OCA or other pigmentation defects, two affect the cytoplasmic domain. The tyrosinase gene in mice homozygous for the e<sup>p</sup> (platinum) allele contains a single base substitution that eliminates all but the basic transmembrane anchor residues of the cytoplasmic tail; the product of this allele is therefore mistargeted and expressed at the cell surface (30). A similar mutation in human tyrosinase results in OCA (31), but the localization of the gene product in melanocytes from these patients has not been characterized. These reports suggest that the cytoplasmic domain of tyrosinase is critical for melanosomal protein sorting. Nevertheless, neither the precise nature of the sorting signals nor their behavior in non-pigmented cells is understood. Here, we explore the possibility that sorting signals with unique characteristics direct tyrosinase into a sorting pathway that is distinct from that used by typical residents of late endosomes and lysosomes.

The cytoplasmic domain of human tyrosinase contains several potential conventional sorting signals. Among these are sequences that conform to classic di-leucine-based (LL) motifs (E<sup>510</sup>KQPLL<sup>515</sup>) and tyrosine-based motifs of the YXXØ consensus sequence (in which a tyrosine, Y, is separated by two residues, X, from an amino acid containing a bulky hydrophobic side chain, Ø; Y<sup>525</sup>HSL<sup>529</sup>), both common among proteins localized to endosomal compartments (32–36). The cytoplasmic domain also contains a second tyrosine-containing sequence (Y<sup>525</sup>QSHL<sup>529</sup>) (see Fig. 1a). All three are conserved in mouse tyrosinase, and the LL motif and Tyr<sup>525</sup> are conserved throughout vertebrate evolution. A role for the LL motif in melanosomal sorting is supported by a requirement of LL residues in tyrosinase related protein-1 (TRP-1) for intracellular localization (37) and by recent evidence that the LL motif from mouse tyrosinase can bind to the AP3 sorting complex in vitro (38). We postulated that some or all of these motifs may direct endosomal sorting in a non-pigmented cell type. Here, using a panel of chimeric proteins and tyrosinase constructs in which these putative sorting signals have been altered by mutagenesis, we characterize the trafficking of tyrosinase in non-pigmented cells.

Our results show that the LL motif is a dominant signal that directs late endosomal sorting through a distinct pathway from that directed by another well characterized late endosomal LL-based signal. This suggests that non-pigmented cells contain a second independent sorting pathway to the late endosome. Such a pathway provides a potential mechanism by which melanocytic cells can differentially sort proteins to the melanosome.

**MATERIALS AND METHODS**

**Antibodies**—Wild-type and mutant forms of human tyrosinase were recognized by aPEP7h (39), a rabbit antiserum to the tyrosinase-related protein-1 (gift of V. Hearing, National Institutes of Health, Bethesda), or NY8K (40), a rabbit antiserum to the lumenal domain (gift of L. Old, Ludwig Institute for Cancer Research, New York). Chimeric proteins containing the lumenal domain of the interleukin-2 receptor α chain (Tac) were detected using the mouse monoclonal antibody (mAb) 7G7.B6 (American Type Culture Collection, Rockville, MD) or a rabbit anti-Tac serum (41). Human invariant chain was detected using mAb 42) to the lumenal domain or PIN.1 (gift of P. Cresswell, Yale University, New Haven, CT; Ref. 43) to the cytoplasmic domain. The following tyrosine-based motif; AP, adaptor-like complex; TRP, tyrosinase-related protein; mAb, monoclonal antibody; IFM, immunofluorescence microscopy; CHX, cycloheximide; FITC, fluorescein isothiocyanate; LRSC, lissamine rhodamine-sulfonyl chloride; PE, phycoerythrin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ER, endoplasmic reticulum.

**FIG. 1.** Wild-type and mutant constructs of tyrosinase. a, schematic representation of the tyrosinase gene product showing the predicted signal sequence (SS), lumenal, transmembrane (TM), and cytoplasmic (Cyt) domains. The sequence of the amino acid residues in each domain are indicated at the top. The amino acid sequence of the predicted wild-type cytoplasmic domain, beginning with the cysteine residue at position 500, is shown below with putative targeting determinants shown in bold. The sequence of the site-directed mutants, named for their amino acid substitution at the bold sites, are shown below. The transmembrane domain is defined as ending in the amino acid sequence LVSLL, b, schematic depiction of the domain structure of Tac/tyrosinase chimeras. The coding region for the cytoplasmic domain of tyrosinase was appended to that for the lumenal and transmembrane domains of Tac using a double PCR technique. The wild-type and mutant amino acid sequences of the cytoplasmic domains of Tac chimeric constructs are listed below. The transmembrane domain of Tac is defined here as ending in the amino acid sequence GLTWQ. c, schematic representation of Tac chimeras TTL1 and Tac-DKQTLL used in degradation and overexpression studies. The sequences of characterized tyrosine- and di-leucine-based sorting signals in the cytoplasmic domains are indicated. The sequence DKQTLL is derived from the CD3γ chain.
Tyrosinase Cytoplasmic Sorting Signals

lists endogenous cellular proteins and reagents used to detect them: lamp1, mAb HA43 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) or a rabbit anti-lamp1 serum (gift of M. Fukuda, Scripps Research Institute, San Diego CA) (44, 45); lamp2, mAb H4B4 (Developmental Studies Hybridoma Bank); transferrin receptor, mAb E104 (Biologics, Inc.); and diphteria toxin receptor, mAb D35 (kind gift of San Rafael, CA). Transient transfections of cells grown on coverslips in 6 well dishes were performed essentially as described (46) using calcium phosphate precipitation. Except for overexpression studies, 50–100 ng of "expressor" plasmid DNA was used, and the balance was made up with "carrier" plasmid (empty pCDM8.1 vector) to 5 μg total; pilot studies showed that this protocol allowed detectable expression of previously characterized lyosomal targeted proteins without significant overexpression, as assessed by surface expression. Cells were generally assayed 2–3 days following transfection. For overexpression and competition experiments, cells were dividing in 16–100-mm dishes and transfected with 8 or 20 μg of DNA, respectively.

Plasmas—The R402Q allele of human tyrosinase was supplied in the mammalian expression vector pCI (Promega, Madison, WI) by W. Storkus (University of Pittsburgh, Pittsburgh, Pennsylvania). Wild-type human tyrosinase 501 (gift of S. Topham, National Cancer Institute, Bethesda), originally inserted into pCDNA3, was cloned into pCDM8.1 using EcoRI and XhoI restriction sites. Original site-directed mutants were constructed using the tyrosinase R402Q construct, but due to apparent misfolding in cells grown at 37 °C (2), the R402Q substitution was corrected and the corrected form was used in all experiments shown. Correction was performed by PCR-mediated amplification of the site-directed mutants of tyrosinase R402Q sequences downstream of the allelic variation (coding region for the remaining lumenal domain fragment and the transmembrane and cytoplasmic domains) and substitution of the amplicon in wild-type tyrosinase 501 cDNA using BglII and XhoI restriction enzymes. The nuclease acid sequence of all constructs was confirmed by dideoxynucleotide sequencing. The plasmids pCDM8.1 (47), human invariant chain p33 (Ip33; Ref. 48), Tac (49) and the chimeric constructs TTMb (46), TTL1 (50), and Tac-DKQTLL (originally referred to as TTMb-t3-t2; Ref. 51), all cloned into pCDM8.1, have been previously described.

Site-directed Mutagenesis—Most site-directed mutants of the cytoplasmic tail of tyrosinase (see Fig. 1a) were constructed using the GeneEditor in vitro Mutagenesis System (Promega, Madison, WI) according to the directions of the supplier (details available upon request). Constructs in which Tyr285 (XX/XA) was mutated were made by PCR-mediated amplification of the wild-type or previously mutated sorting signals followed by sub-cloning of the amplicon using a unique BglII recognition site in the tyrosinase coding region and an XhoI restriction site in the vector. Δtail-1 and Δtail-2 were made by insertion of a single PCR amplicon from the original construct. Plasmid DNA was screened for a unique restriction enzyme fragment and the transmembrane and cytoplasmic domains) and substitution of the amplicon into wild-type tyrosinase 501 cDNA using EcoRI and XhoI restriction sites. Original site-directed mutants were constructed using the tyrosinase R402Q construct, but due to apparent misfolding in cells grown at 37 °C,2 the R402Q substitution was corrected and the corrected form was used in all experiments shown. Correction was performed by PCR-mediated amplification of the site-directed mutants of tyrosinase R402Q sequences downstream of the allelic variation (coding region for the remaining lumenal domain fragment and the transmembrane and cytoplasmic domains) and substitution of the amplicon into wild-type tyrosinase 501 cDNA using BglII and XhoI restriction enzymes. The nuclease acid sequence of all constructs was confirmed by dideoxynucleotide sequencing. The plasmids pCDM8.1 (47), human invariant chain p33 (Ip33; Ref. 48), Tac (49) and the chimeric constructs TTMb (46), TTL1 (50), and Tac-DKQTLL (originally referred to as TTMb-t3-t2; Ref. 51), all cloned into pCDM8.1, have been previously described.

Construction of Tac Chimeras—All Tac chimeric constructs were prepared as described (46) in the mammalian expression vector pCDM8.1. Tac chimeras were generated for all constructs listed in Fig. 1 using a two-step PCR method (52). Briefly, a chimeric insert was made encoding the luminal and transmembrane domains of Tac fused to the cytoplasmic domain of tyrosinase flank by unique BglII and XhoI restriction sites. The chimeric insert was then ligated into a previously modified Tac chimeric construct. DNA sequences of all chimeric constructs were confirmed by dideoxynucleotide sequencing.

Immunofluorescence Microscopy—Forty-eight hours following transfection, cells were fixed with 2% formaldehyde in PBS for 20 min at room temperature and washed with PBS. In some cases, cells were treated with Cycloheximide (CHX; 50 μg/ml) for 1 h in fresh Dulbecco’s modified Eagle’s medium and washed in PBS prior to fixation. Cells that had been transfected with DNA encoding Tac chimeric proteins were either untreated or treated with 1 mg/ml leupeptin for 4 h prior to fixation. Cells were stained as described (53). Stained coverslips were mounted on microscope slides using Fluoromount-G (Southern Biotechnologies Associates, Birmingham, AL) and visualized by fluorescence microscopy using a Zeiss Axiovert microscope. Photographic images were digitized from negatives using a Nikon LS-1000 slide scanner and Adobe Photoshop 3.0 software (Adobe Systems, San Rafael, CA). Time-lapse fluorescence microscopy studies and measurements of late endosomal diameter, analyses were done using a Leica microscope fitted with a MicroMax digital camera (Princeton Instruments Inc., Trenton, NJ) and OpenLab software (Improvision, Coventry, UK). Statistical analyses of endosomal diameter measurements was performed using the Mann-Whitney Rank Sum Test on SigmaStat software (Abacus Concepts). Average expression per cell was calculated by dividing the total radioactivity in precipitable bands by the transfection efficiency (fraction of positively stained cells by immunofluorescence microscopy).

Antibody Internalization Assay—HeLa cells transiently transfected with Tac chimeras on coverslips were incubated with purified 7G7.B6 anti-Tac antibody (50 μg/ml) in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum for 30 min at 37 °C. All coverslips were washed 3 times in PBS and fixed for 20 min in 2% formaldehyde in PBS. Internalized mAb 7G7.B6 was detected using LRSC-conjugated goat anti-mouse Ig, and total cellular Tac chimeras were visualized by staining with rabbit anti-Tac and FITC-conjugated goat anti-rabbit Ig as described above.

Sorting Signal Competition Assay—This was performed essentially as described (50). Tac chimeric proteins were purposefully overexpressed in HeLa cells by transient transfection using 20 μg plasmid DNA encoding Ip33 as a reporter protein. Sixty hours post-transfection, cells were harvested and divided into aliquots for analyses. In some experiments, levels of expression from each of the transfected DNAs was determined by pulse metabolic labeling of 1 aliquot followed by immunoprecipitation. For flow cytometric analysis, 0.5–10% viable cells were stained with the indicated primary antibodies for 1 h, washed, and then stained with FITC- and PE-conjugated secondary antibodies for an additional hour. Cells were then washed, fixed in 2% formaldehyde in PBS, and analyzed using a Becton Dickinson FACScan (Franklin Lakes, NJ) and CellQuest 1.2 software. Dead cells were excluded from analysis by forward and side scatter measurements. In the analyses shown, cells were gated for those expressing high levels of the "overexpressor" Tac molecule at the cell surface. Transfection efficiencies were monitored by parallel immunofluorescence microscopy analyses.

RESULTS

Tyrosinase Localizes to Late Endosomes and Lyosomes in HeLa Cells—In order to assess the potential function of conventional sorting signals in the cytoplasmic domain, it was first necessary to determine the ultimate destination of tyrosinase in non-pigmented cells. To this end, tyrosinase was expressed in HeLa cells by transient transfection, and its localization was assessed by indirect immunofluorescence microscopy (IFM).

When expressed at moderate levels, tyrosinase was detected in the nuclear envelope and in reticular and vesicular structures both throughout the cell cytoplasm and in the perinuclear area in most transfected cells (Fig. 2a). Reticular and nuclear envelope staining was variegated and more intense at higher levels of overexpression (data not shown). The overexpression pattern would be consistent with localization to both the endoplasmic reticulum (ER) and endosomal compartments at steady state. Prominent ER staining is likely a result of slow egress of tyrosinase from the ER due to inefficient folding, supported by pulse/chase analyses in which the half-time for acquisition of resistance to digestion with endoglycosidase H is longer than
were double-stained with antibodies to tyrosinase and to enzymes of lysosomal protease inhibitors, suggesting that it is relatively stable to proteolysis within these compartments. To determine the identity of the structures, CHX-treated transfected cells were double-stained with antibodies to tyrosinase and to endogenous endosomal proteins. As shown in Fig. 3, a and b, tyrosinase colocalized with lamp1, a constituent of late endosomes and lysosomes. The staining pattern with both markers was nearly identical in most cells at various expression levels. Near perfect colocalization was also observed with another marker of late endosomes and lysosomes, lamp2, but not with transferrin receptor (early and recycling endosomes), and peripheral tyrosinase vesicular structures did not contain the cation-independent mannose 6-phosphate receptor (trans-Golgi network/late endosomes; data not shown). The staining pattern for lamp1 in cells that expressed the transgene did not consistently differ from that in cells that were not transfected, indicating that tyrosinase expression at moderate levels does not alter the morphology of lamp1-positive compartments. We conclude that tyrosinase is directed to conventional late endosomes and lysosomes in HeLa cells. Similar observations were made in CV-1, M1, and 293 cell lines (data not shown).

Role of Di-leucine and Tyrosine-based Sorting Signals in Localization of Tyrosinase to Late Endosomes and Lysosomes—To characterize the cytoplasmic sequences responsible for targeting tyrosinase to endosomal compartments, we used site-directed mutagenesis to disrupt critical residues of the putative LL and YXXØ sorting motifs and the additional conserved tyrosine residue Tyr525. Critical residues were altered individually or in combination to alanine (Fig. 1a). Each mutagenized construct was expressed in HeLa cells by transient transfection, and localization was assessed in cells treated with CHX for 1 h by IFM and double staining with antibodies to the tyrosinase transgene and to endogenous lamp1.

Tyrosinase with either or both of the cytoplasmic tyrosine residues disrupted (LL/AN, LL/YA, and LL/A/A) demonstrated IFM staining patterns similar to that of intact tyrosinase with either wild-type (a and b) or mutated (c–f) cytoplasmic domains and were analyzed 2 days following transfection by intracellular IFM using a rabbit antiserum to tyrosinase (left), HA3 to detect endogenous lamp1 (right), and FITC- and LRSC-conjugated secondary antibodies. Prior to fixation, all cells were treated with 50 μg/ml CHX for 1 h at 37 °C. Filled arrowheads point to examples of vesicles in which both tyrosinase and lamp1 were detected. Arrows point to examples of vesicles in which only one protein, but not the other, was stained. Open arrowheads point to larger vesicles with outer rim staining in cells expressing tyrosinase with mutations at Tyr525 (g–j).

To visualize more clearly post-ER compartments, we therefore reduced the pool of newly synthesized, ER-retained tyrosinase by treating transfected cells with the protein synthesis inhibitor, cycloheximide (CHX).

As shown in Fig. 2b, the staining pattern of cells treated for 1 h with CHX was predominantly vesicular. The appearance of tyrosinase in vesicular structures did not require the addition of lysosomal protease inhibitors, suggesting that it is relatively stable to proteolysis within these compartments. To determine the identity of the structures, CHX-treated transfected cells were double-stained with antibodies to tyrosinase and to endogenous endosomal proteins. As shown in Fig. 3, a and b, tyrosinase colocalized with lamp1, a constituent of late endosomes and lysosomes. The staining pattern with both markers was nearly identical in most cells at various expression levels. Near perfect colocalization was also observed with another marker of late endosomes and lysosomes, lamp2, but not with transferrin receptor (early and recycling endosomes), and peripheral tyrosinase vesicular structures did not contain the cation-independent mannose 6-phosphate receptor (trans-Golgi network/late endosomes; data not shown). The staining pattern for lamp1 in cells that expressed the transgene did not consistently differ from that in cells that were not transfected, indicating that tyrosinase expression at moderate levels does not alter the morphology of lamp1-positive compartments. We conclude that tyrosinase is directed to conventional late endosomes and lysosomes in HeLa cells. Similar observations were made in CV-1, M1, and 293 cell lines (data not shown).

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that contribute to sorting of tyrosinase, we transferred the cytoplasmic domain alone to a reporter protein, Tac. Tac (the human interleukin-2 receptor α chain) is a monomeric, cell surface, type I integral membrane protein that we have previously used as a reporter to characterize a number of post-Golgi sorting signals (10, 41, 46, 50, 54). A Tac/tyrosinase (TTY) chimera was generated containing the lumenal and transmembrane domains of Tac and the cytoplasmic domain of tyrosinase (Fig. 1b); additional Tac chimeric proteins contained the site-directed mutations of the tyrosinase cytoplasmic domain (Fig. 1b). HeLa cells were transiently transfected with each of the Tac chimeric constructs, and the expressed products were detected by IFM. Since the Tac epitopes detected by our antibodies are sensitive to proteolysis in late endosomes and lysosomes (46, 50, 51), cells were examined after a 4-h treatment with leupeptin.

The results show that TTY behaved similarly to tyrosinase and was localized to vesicular structures that overlapped nearly completely with structures containing lamp1 (Fig. 4, a and b). Furthermore, cells co-transfected with both TTY and tyrosinase and treated with leupeptin demonstrated nearly complete overlap of the two transgenes (data not shown). The staining pattern of TTY was largely dependent on treatment of cells with leupeptin (e.g. see Fig. 5), consistent with degradation of the Tac lumenal domain in proteolytic late endosomal compartments as implicated by parallel pulse/chase experiments (data not shown). The localization of Tac chimeric proteins with altered cytoplasmic tail residues paralleled that of the corresponding tyrosinase mutant; disruption of the LL motif resulted in cell-surface expression (Fig. 4, c and d), whereas disruption of either (not shown) or both tyrosine residues had no or subtle effects (Fig. 4, e and f).

Residual vesicles associated with the altered LL motif mutants (TTY-AA/Y/Y and TTY-AA/Y/A) often colocalized with lamp1 (Fig. 4, c and d, and data not shown). Although this result could indicate the existence of a secondary late endosomal sorting signal, the absence of such colocalization with the corresponding full-length tyrosinase constructs would support the alternative conclusion that these vesicles represent the products of normal cell-surface protein turnover made more apparent by leupeptin treatment. Unmodified Tac was expressed only at the cell surface under these conditions (see Fig. 5), and chimeric proteins containing the lumenal or transmembrane domains of tyrosinase without the transmembrane domain were not localized to endosomal compartments (data not shown), suggesting that only the cytoplasmic domain contains independent endosomal sorting information. These data demonstrate that the cytoplasmic domain of tyrosinase is both necessary and sufficient for localization to conventional late endosomes and lysosomes in HeLa cells, and these data confirm the role of the LL motif in efficient endosomal protein sorting.

The Di-leucine Motif of Tyrosinase Functions as an Internalization Signal at the Plasma Membrane—Most post-Golgi sorting signals also serve as internalization signals that function in efficient retrieval of intracellular proteins from the cell surface. To determine whether the tyrosinase cytoplasmic sorting signals function similarly, we used IFM to assess the ability of the TTY chimeric proteins to effect efficient internalization of anti-Tac antibody in live cells. Chimeric proteins were transiently expressed in HeLa cells at moderate levels, and anti-Tac mAb 7G7.B6 was added to the medium for 30 min at 37 °C to allow antibody uptake prior to fixation. Internalized antibody was detected by staining with anti-mouse immunoglobulin, and total cellular Tac chimeric protein was visualized using a rabbit anti-Tac and FITC anti-rabbit Ig (Total Tac chimera). Note that cells were not treated with leupeptin in this experiment.

FIG. 5. The di-leucine motif of tyrosinase functions as an internalization signal. HeLa cells were transiently transfected with plasmids encoding TTY chimeric proteins, Tac, or TTL1 as indicated. Two days later, cells were incubated with 50 μg/ml purified mAb 7G7.B6 in complete medium at 37 °C, and the antibody was allowed to internalize for 30 min. Cells were then fixed and permeabilized, and internalized antibody was detected by IFM using LRS-conjugated anti-mouse Ig (Int. 7G7.B6). Transfected cells were identified by counterstaining permeabilized cells with rabbit anti-Tac and FITC anti-rabbit Ig (Total Tac chimera).
ent effect on internalization relative to the wild type (Fig. 5, e and f and data not shown). In contrast, disruption of the LL residues dramatically reduced internalization, as evidenced by the intense cell-surface staining from 7G7.B6 in cells transfected with either TTY-AA/Y/Y or TTY-AA/Y/A (Fig. 5, c and d and data not shown). Interestingly, there was a small degree of vesicular 7G7.B6 staining with both of these chimeras that was not visible in cells transfected with Tac alone (Fig. 5, g and h), implicating the existence of a weak internalization signal distinct from the LL motif in the cytoplasmic domain. Taken together, these data suggest that the LL motif in the cytoplasmic domain of tyrosinase is part of a strong internalization signal, and secondary signals may also contribute to internalization. They further imply that tyrosinase may pass through the plasma membrane at some point during its trafficking.

The Di-leucine Signal of Tyrosinase Interacts with Saturable Components Involved in Sorting Mediated by Other Di-leucine- and Tyrosine-based Signals—By using transient overexpression and a cell-surface displacement assay, we and others (41, 50, 55, 56) have shown that conventional sorting signals that conform to a given motif utilize common saturable components to effect localization but that signals conforming to different motifs utilize distinct saturable components. If the sorting signals in the tyrosinase cytoplasmic domain corresponded to conventional signals, then it would be expected that they would compete for binding sites with endogenous proteins bearing similar signals. Therefore, overexpression of the tyrosinase cytoplasmic domain would result in mis-sorting and displacement of these endogenous proteins to the cell surface. We tested this hypothesis by overexpressing Tac chimeric proteins containing either well described, conventional sorting signals or the cytoplasmic domain of tyrosinase and using flow cytometry to analyze the cell-surface displacement of endogenous or weakly expressed exogenous proteins containing LL or XXXØ motifs. Levels of expression of each Tac chimeric protein were monitored by metabolic labeling and immunoprecipitation, and transfection efficiencies were monitored by IFM.

HeLa cells were transfected with sufficient levels of plasmid DNA encoding Tac chimeric proteins to saturate sorting pathways and appear on the surface of a large fraction of cells. To assay for competition with LL motifs, cells were co-transfected with low levels of plasmid DNA encoding the p33 form of human invariant chain (Ip33). Ip33 contains two well characterized LL-like lysosomal targeting signals in its cytoplasmic domain, DQRDLI and EQLPMI (57–60). As shown in Fig. 6a, overexpression of TTY caused displacement of Ip33 to the cell surface at levels comparable to those induced by overexpression of Tac appended with the LL motif from the CD33 chain (Tac-DKQTLL; Fig. 6b). As previously shown (50), overexpression of Tac containing no targeting signals (Tac; Fig. 6c) or a functional XXXØ motif (TTL1; Fig. 6d) did not induce significant surface expression of Ip33. Mutagenesis of Tyr521 (TTY-LL/A/Y) did not affect the ability of TTY to displace Ip33 to the cell surface (Fig. 6c). In contrast, alteration of the LL signal (TTY-AA/Y/Y) abolished competition to levels comparable with Tac (Fig. 6c). These data indicate that the LL motifs in the cytoplasmic domains of tyrosinase and Ip33 use common saturable components to effect localization.

To assay for competition with XXXØ-like sorting signals, we analyzed transfected cells for the cell-surface appearance of two endogenous lysosomal membrane proteins, lamp1 and lamp2. Both proteins rely on well characterized tyrosine-based sorting signals (AGYQTL in lamp1 and AGYEQF in lamp2) to effect late endosomal localization. As shown in Fig. 7, a and d, overexpression of TTY results in significant displacement of both endogenous lamp1 and lamp2 to the cell surface. This displacement is much greater than that observed upon overexpression of Tac-DKQTLL but not as substantial as that induced by overexpression of TTL1; this suggests either a weak affinity for a XXXØ recognition site, a partial steric block to such a site, or competition for a distinct and somewhat less limiting factor. Remarkably, disruption of the LL motif (AA/Y/Y) completely eliminated surface displacement of both lamp1 and lamp2 (Fig. 7, b and e) to levels comparable to that induced by overexpression of Tac with no signal. Thus, competition for XXXØ-based sorting is dependent on the integrity of the tyrosinase LL residues. Disruption of both tyrosines in the tyrosinase cytoplasmic domain (TTY-LL/A/A) reduced, but did not eliminate, cell-surface displacement of both lamp1 and lamp2 (Fig. 7, b...
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Fig. 7. Disruption of sorting mediated by tyrosine-based motifs by overexpression of TTY. HeLa cells were transiently transfected with saturating levels (20 μg/10 cm dish) of plasmids encoding Tac, the indicated Tac chimeras, or a control (not shown). Three days after transfection, cells were harvested and stained for surface expression with a rabbit antibody to Tac and mouse monoclonal antibodies to endogenous lamp1 (a–c) or lamp2 (d–f), and then FITC-anti-mouse Ig and PE-anti-rabbit Ig. Stained cells were analyzed by flow cytometry. Shown is a representative of four experiments, and data are presented as in Fig. 6. In the experiment shown, TTY, LL/Y/A, LL/A/Y and LL/A/A were expressed at comparable levels and about 2-fold higher than Tac, AA/Y/Y, or Tac-DKQTLL; TTL1 was expressed about half as well as these. In other experiments with similar results, expression levels for all constructs were comparable.

and e), suggesting that tyrosine residues influence the ability of the cytoplasmic domain to compete with YXXØ signals. However, the same level of displacement was observed if either tyrosine residue was individually disrupted (LL/Y/A, LL/A/Y; Fig. 7, c and f). This suggests either that both tyrosines take part in a novel sorting signal that weakly or partially competes for cellular factors with conventional YXXØ motifs or that each tyrosine influences the context of the LL motif such that it is now able to block access of cellular components to YXXØ motifs. It further suggests that HeLa cells retain the ability to distinguish structural features of sorting signals in the cytoplasmic domain of tyrosinase from other conventional endosomal sorting signals.

Enlargement of Late Endosomes and Lysosomes by Overexpression of the Di-leucine Signal of Tyrosinase but Not of CD3γ—When cells overexpressing TTY were analyzed by IFM, the TTY product was observed to accumulate not only at the cell surface but also in the periphery of large vesicular structures, the lumen of which was clearly visible (Fig. 8a). Comparison of lamp1 staining revealed a concomitant enlargement of lamp1-positive compartments in cells overexpressing TTY relative to untransfected cells (Fig. 8b). Similarly enlarged lamp1-positive structures were observed in cells overexpressing full-length tyrosinase (Fig. 8, i and j). These structures accumulated more in the perinuclear area than in distal regions of the cell. Measurement of lamp1-positive structures in untransfected cells or cells expressing high levels of TTY at the cell surface revealed a 2-fold increase in diameter in the latter cells (1.026-μm median diameter), representing a 4-fold increase in surface area, relative to the untransfected cells (0.498 μm median diameter; see Table I). This difference was statistically significant (p < 0.0001). Enlargement of lamp1-positive compartments was also observed upon overexpression of TTY-LI/A/A (Fig. 8, e and f), TTY-LI/Y/A and TTY-LI/A/Y (not shown), but not of TTY-AA/Y/Y (Fig. 8, c and d) or tyrosinase-AA/Y/Y (Fig. 8, h and j), indicating that the enlargement was dependent on the integrity of the LL motif but not of either or both tyrosine residues. Importantly, overexpression of comparably high levels of Tac-DKQTLL, with the LL motif from CD3γ, failed to induce a dramatic enlargement of lamp1-positive structures (0.573 μm median diameter), although there was a statistically significant difference in comparison to untransfected cells (0.498 μm median diameter; p < 0.0001) (Fig. 8, g and h, and Table I). The massive enlargement observed upon overexpression of TTY is likely due to disruption of sorting of a protein(s) required for maintenance of late endosomal volume or surface area. These results suggest that the LL signal of tyrosinase mediates sorting through a common pathway with such a protein, whereas the LL signal of CD3γ does not. We conclude, therefore, that these two signals direct sorting to late endosomes via distinct pathways.

DISCUSSION

The mechanisms by which proteins are localized to tissue-specific organelles are poorly understood, but in many cases they may take advantage of existing redundant pathways to ubiquitous organelles. We (10) and others (9, 11–13) have previously described evidence that multiple pathways exist for protein transport to late endosomes and lysosomes, providing the potential for subversion of one pathway for localization to tissue-specific late endosomal organelles. Here, we have extended these studies to demonstrate that a distinct type of LL-based late endosomal/lysosomal sorting signal exists in the cytoplasmic domain of tyrosinase, an integral membrane protein component of the melanosome. The singular behavior of the tyrosinase sorting signals in non-pigmented cells provides a potential mechanism by which these signals might be distinguished for localization to the unique, late endosome-like melanosome in melanocytic cells.

Tyrosinase Localizes to Late Endosomes and Lysosomes of Non-pigmented Cells—The cytoplasmic domain of tyrosinase proved to be both necessary and sufficient for localization to conventional late endosomes and lysosomes of non-pigmented cells, suggesting that it contains a classical late endosomal/lysosomal sorting determinant. This was evidenced by nearly
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complete colocalization of full-length tyrosinase or the TTY chimeric protein with lamp1 and lamp2 in transfected cells, and by predominant cell-surface expression upon mutagenesis of the LL motif or deletion of the cytoplasmic domain. Our immunolocalization data extend previous observations in which products of tyrosinase activity co-fractionated with lysosomes or lysosome-like structures in transfected cells (Refs. 61–63 and reviewed in Refs. 21 and 23). A requirement for the cytoplasmic domain in proper localization is consistent with the trafficking defects of the murine C allele (30), the OCA phenotype of human patients with a mutation rendering loss of the cytoplasmic domain (31), and properties of the cytoplasmic domain of the tyrosinase-related protein TRP-1 (37).

Like other classical late endosomal sorting signals, the cytoplasmic domain of tyrosinase also directed internalization and late endosomal localization from the plasma membrane, since anti-Tac antibody was internalized and delivered to vesicular compartments in cells expressing TTY. This is the first described indication that melanosomal proteins may be internalized and is consistent with previously reported observations that a fraction of TRP-1 resides at the plasma membrane (64) and that endocytosed albumin-gold conjugates can be found in melanosomes (22). If the cytoplasmic domain of tyrosinase functions similarly in cells of the melanocytic lineage, such recycling might serve to preserve cellular tyrosinase levels in the face of ongoing secretion of melanin.

A Second Class of Di-leucine-based Sorting Signals—A role for LL-based signals in melanosomal protein localization was first implied by the loss of intracellular localization of the tyrosinase-related protein, TRP-1, in fibroblasts and melanocytes upon carboxyl-terminal truncation that eliminated a sequence overlapping a LL-like signal (37). In vitro binding of a LL motif from the mouse tyrosinase cytoplasmic domain to the adaptor-like protein complex, AP3, lent further indirect support to a critical role for LL signals (38). Our point mutagenesis studies clearly showed that efficient internalization and steady state localization of human tyrosinase to late endosomes and lysosomes is dependent on two consecutive leucine residues that lie within a sequence conforming to a classical LL motif (35, 51, 65). Somewhat surprisingly, mutagenesis of either of two conserved tyrosine residues (Tyr521 or Tyr525), one of which (Tyr521) lies within a consensus YXXØ motif, had only minimal effects on steady state localization and internalization. Furthermore, these residues were unable to sustain localization of full-length tyrosinase to late endosomes or lysosomes in the absence of a functional LL motif. These data show that the LL residues of tyrosinase, but not the tyrosine residues, are critical components of a sorting determinant for late endosomes and lysosomes.

The results of an in vivo competition assay suggest that the critical LL residues form the foundation for a LL motif-based sorting signal but that this signal interacts either with unique limiting cellular components or with common LL-binding components in a unique manner. In this assay, overexpression of TTY resulted in extensive displacement from intracellular stores to the cell surface of the invariant chain, a protein that relies on two LL-like motifs for its sorting to late endosomes (57–59). This displacement was as effective as that achieved upon overexpression of a chimeric protein containing the LL signal from CD3γ (51) and was much greater than that observed with the YXXØ signal from lamp1 as previously shown (50). We conclude from these results that TTY effectively competes for limiting cellular factors in sorting and internalization mediated by well characterized LL motifs. However, overexpression of TTY also resulted in significant cell-surface displacement of lamp1 and lamp2, two proteins that rely on YXXØ signals for lysosomal sorting and internalization. This displacement was not as great as that induced by overexpression of the lamp1 YXXØ signal, but was much greater than that induced by overexpression of Tac alone or of the LL signal from CD3γ. We interpret this displacement as an indication that TTY partially competes for limiting cellular factors involved in either

| Overexpressed construct | Median length (sample number) | 25th and 75th percentile values |
|-------------------------|------------------------------|--------------------------------|
| TTY                     | 1.026 (n = 113)              | 0.904–1.113                     |
| Tac-DKQTL                | 0.573 (n = 193)              | 0.477–0.660                     |
| Untransfected           | 0.498 (n = 125)              | 0.441–0.555                     |
the recognition of YXXØ signals or sorting and internalization mediated by these signals. Importantly, induction of lamp1 and lamp2 surface expression was ablated by mutagenesis of the LL residues, indicating that competition for YXXØ signals was dependent on the integrity of the LL motif. This interaction with YXXØ-based recognition/sorting factors represents a novel property of a LL-based sorting signal, since competition for YXXØ motifs is not observed with the CD3y LL signal (here and Ref. 50). The data therefore suggest that the tyrosinase LL-based signal interacts with at least partially distinct factors to direct sorting to late endosomes and lysosomes in HeLa cells.

A second indication of the distinctive nature of the tyrosinase sorting signal came from IFM analysis of overexpressing cells. Overexpression of TTY or tyrosinase induced enlargement of lamp1-positive late endosomes and lysosomes in HeLa cells, whereas expression of comparably high levels of Tac-DKQTLL (with the CD3y LL signal) had no such effect. This effect was dependent on the LL residues of the tyrosinase cytoplasmic domain but not the distal tyrosine residues. The enlargement could be due to either accumulation of luminal contents (failure to degrade lumenal contents), disruption of ion transport across the late endosomal membrane (increased luminal ionic strength to cause swelling), or failure to form vesicles from the limiting membrane (inhibition of budding), any of which most likely results from competitive inhibition of a specific sorting step for a protein critical in each process by overexpression of the tyrosinase, but not the CD3y, LL signal. Interestingly, similar observations have been made upon overexpression of the invariant chain (66, 67). The sequence of the invariant chain (66, 67). The sequence of the membrane proximal of the two LL-like signals in invariant chain is similar to that of tyrosinase (57, 59, 60, 68), suggesting that both proteins may be sorted by similar mechanisms.

**Influence of Tyrosine Residues on Context of the Di-Leucine Signal**—Although the tyrosine residues Tyr²⁹¹ and Tyr²⁹⁵ do not appear to contribute significantly to signals with intrinsic sorting activity in HeLa cells, our competition results suggest that they may contribute to the context in which the LL motif is recognized. This was evident by the partial reduction in the ability of overexpressed TTY to displace lamp1 and lamp2 to the surface upon mutagenesis of either Tyr²⁹¹, Tyr²⁹⁵, or both. The partial but additive effects of these mutations on lamp1 and lamp2 surface expression was ablated by mutagenesis of the LL residues, indicating that competition for YXXØ signals was dependent on the integrity of the LL motif. This interaction with YXXØ-based recognition/sorting factors represents a novel property of a LL-based sorting signal, since competition for YXXØ motifs is not observed with the CD3y LL signal (here and Ref. 50). The data therefore suggest that the tyrosinase LL-based signal interacts with at least partially distinct factors to direct sorting to late endosomes and lysosomes in HeLa cells.

**Implications for Melanosomal Protein Sorting**—We (data not shown) and others (reviewed in Refs. 21 and 23) have observed differential localization of lamp1 and tyrosinase in some melanocytes and melanoma cells, suggesting that melanosomal and late endosomal integral membrane proteins are differentially recognized by the melanocyte protein sorting machinery. Our results here provide a potential mechanism to account for this differential recognition. It is conceivable that the cell surface receptors that interact with the LL signal from tyrosinase and facilitate sorting via a unique pathway to late endosomes in HeLa cells may be usurped by cells of the melanocyte lineage to direct proteins such as tyrosinase and TRP-1 to melanosomes. Sequence comparison of tyrosinase, TRP-1, and TRP-2 from several species shows a striking conservation of general structural features of the cytoplasmic domain that would be consistent with recognition by a distinct sorting receptor (Fig. 9). First, each of these proteins contains a LL- (tyrosinase and TRP-1) or YXXØ-based (TRP-2) late endosomal/lysosomal sorting signal at a conserved position close to the membrane. Second, each protein, particularly tyrosinase, has unusually high content of basic residues in the membrane proximal region, aminoterminal to the lysosomal sorting signal. These residues could interact with acidic headgroups of phospholipids and/or with a third conserved region of the cytoplasmic domains rich in acidic amino acid residues and distal to the conventional sorting signal. If these two regions were to interact three-dimensionally, an unoccupied, conserved tyrosine residue, analogous to Tyr²⁹⁵ in human tyrosinase, could be placed in close proximity to the conventional sorting signal where it might be able to affect interactions with APs. Although highly speculative, this model creates a conceptual framework with which to test functions of different regions of the cytoplasmic domains of tyrosinase, TRP-1, and TRP-2 in melanosomal protein sorting.

It is particularly intriguing that a determinant capable of

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**Table: Tyrosinase Cytoplasmic Sorting Signals**

| Protein                        | YXXØ Signal | Acidic/Spacer | Tyrosine/Acidic |
|--------------------------------|-------------|---------------|-----------------|
| human tyrosinase               |             |               |                 |
| mouse tyrosinase               |             |               |                 |
| chicken tyrosinase             |             |               |                 |
| frog tyrosinase                |             |               |                 |
| fish tyrosinase                |             |               |                 |
| human TRP2                     |             |               |                 |
| mouse TRP2                     |             |               |                 |
| chicken TRP1                   |             |               |                 |
| salamander TRP1                |             |               |                 |
| fish TRP1                      |             |               |                 |

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**Figure 9: Sequence alignment of the cytoplasmic domains of tyrosinase and tyrosinase-related proteins from different species.** Shown are the deduced amino acid sequences of the predicted cytoplasmic domains from the indicated proteins, aligned into regions as defined in the text. Basic residues within the membrane proximal region are indicated in bold; consensus sorting signals are underlined; acidic residues in the distal region are shaded, and the conserved tyrosine residue is boxed. Sequences were obtained from the GenBank databases.

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**Note:** The table and figure provide a comprehensive alignment of the cytoplasmic domains of tyrosinase and related proteins, highlighting the conserved amino acid residues that are critical for sorting signals and interactions with cellular factors.
mediating specialized localization in pigmented cells effects localization to conventional late endosomes and lysosomes in the inappropriate cell type. The cell type-specific appropriation of a secondary ubiquitous sorting pathway for a specific localization pattern is well established. Proteins that are differentially sorted to basolateral and apical surfaces in polarized cells are delivered to the same cell-surface domain of fibroblasts in separate vesicles (14–16). Furthermore, yeast use two pathways for protein delivery to the vacuole, including one that is dependent on recognition of LL-like motifs by the homologue of AP3 (1). Thus, further understanding of the mechanisms of melanosomal protein sorting should provide insights into general pathways of transport to specialized late endosomal compartments.

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Note Added in Proof—During review of this manuscript, Simmen et al. (Simmon, T., Schmidt, A., Hunziker, W., and Beermann, F. (1999) J. Cell Sci. 112, 45–53) published a characterization of cytoplasmic domain signals required for lysosomal targeting of murine tyrosinase in transfected MDCK cells. They found a similar requirement for the LL domain signals required for lysosomal targeting of murine tyrosinase in transfected MDCK cells. They feel that the data are consistent with our interpretation of transport to specialized late endosomal compartments.

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