

Intracellular Sorting and Targeting of Melanosomal Membrane Proteins: Identification of Signals for Sorting of the Human Brown Locus Protein, GP75

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Abstract. The structural and functional integrity of cytoplasmic organelles is maintained by intracellular mechanisms that sort and target newly synthesized proteins to their appropriate cellular locations. In melanocytic cells, melanin pigment is synthesized in specialized organelles, melanosomes. A family of melanocyte-specific proteins, known as tyrosinase-related proteins that regulate melanin pigment synthesis, is localized to the melanosomal membrane. The human brown locus protein, tyrosinase-related protein-1 or gp75, is the most abundant glycoprotein in melanocytic cells, and is a prototype for melanosomal membrane proteins. To investigate the signals that allow intracellular retention and sorting of glycoprotein (gp)75, we constructed protein chimeras containing the amino-terminal extracellular domain of the T lymphocyte surface protein CD8, and transmembrane and cytoplasmic domains of gp75. In fibroblast transfectants, chimeric CD8 molecules containing the 36-amino acid cytoplasmic domain of gp75 were retained in cytoplasmic organelles. Signals in the gp75 cytoplasmic tail alone, were sufficient for in-

tracellular retention and targeting of the chimeric proteins to the endosomal/lysosomal compartment. Analysis of subcellular localization of carboxyl-terminal deletion mutants of gp75 and the CD8/gp75 chimeras showed that deletion of up to 20 amino acids from the gp75 carboxyl terminus did not affect intracellular retention and sorting, whereas both gp75 and CD8/gp75 mutants lacking the carboxyl-terminal 27 amino acids were transported to the cell surface. This region contains the amino acid sequence, *asn-gln-pro-leu-leu-thr*, and this hexapeptide is conserved among other melanosomal proteins. Further evidence showed that this hexapeptide sequence is necessary for intracellular sorting of gp75 in melanocytic cells, and suggested that a signal for sorting melanosomal proteins along the endosomal/lysosomal pathway lies within this sequence. These data provide evidence for common signals for intracellular sorting of melanosomal and lysosomal proteins, and support the notion that lysosomes and melanosomes share a common endosomal pathway of biogenesis.

BIOGENESIS of cytoplasmic organelles requires targeting of soluble and membrane proteins to appropriate vesicular precursors. Signals responsible for intracellular sorting and targeting of newly synthesized proteins to various organelles have been identified, including those for lysosomes and peroxisomes. Melanosomes, the site of melanin pigment synthesis, are specialized organelles present in melanocytic cells that produce pigment. Biogenesis of melanosomes is not well understood (Novikoff et al., 1968; Maul and Brumbaugh, 1971). Based on electron microscopic histochemical studies, it has been proposed that tyrosinase, the critical and rate-limiting enzyme for melanin synthesis, is transported by coated vesicles from Golgi to premelanosomal vesicles (Novikoff et

al., 1968; Chakraborty et al., 1989; Moellmann et al., 1989; Zhou et al., 1993). However, signals responsible for sorting tyrosinase and other melanosomal membrane proteins for intracellular retention and targeting to premelanosomal vesicles have not been investigated.

Recently, several proteins that influence melanin pigmentation have been identified. These pigmentation-associated proteins include a family of proteins known as tyrosinase-related proteins (TRPs)¹ that are encoded by genes that map to different genetic loci that determine coat color of mice (Shibahara et al., 1986; Jackson, 1988; Vijayasaradhi et al., 1990; Kwon et al., 1991; Gardner et al., 1992; Jackson et al., 1992; Tsukamoto et al., 1992; Rinchik et al., 1993). The product of the *brown* (*b*) locus gene, is a 75-kD

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1. *Abbreviations used in this paper:* Endo H, endoglycosidase H; gp, glycoprotein; lgps, lysosomal glycoproteins; TM, transmembrane; TRP, tyrosinase-related protein.

glycoprotein known as TRP-1 or glycoprotein (gp)75. The *b* locus protein catalyzes the oxidation of 5,6-dihydroxyindole-2-carboxylic acid, an intermediate in the melanin synthesis pathway, and mutations and allelic differences at the *b* locus produce shades of brown coat color in mice (Silvers, 1979; Zdarsky et al., 1990; Jiménez-Cervantes et al., 1994). The *b* locus is of particular interest because it is known to influence the structure of melanosomes (Moyer, 1963, 1966; Foster, 1965; Rittenhouse, 1968; Hearing et al., 1973).

The *b* locus product, gp75, is highly conserved between mouse and human, and shares a 43% amino acid sequence identity with tyrosinase. In human melanocytic cells, gp75 is the most abundant glycoprotein (Vijayasradhi et al., 1991; Tai et al., 1983). The biosynthesis and intracellular movement of gp75 has been studied in detail. gp75 is synthesized as a 55-kD polypeptide, glycosylated by addition and processing of at least five Asn-linked oligosaccharides through the *cis*- and *trans*-Golgi complex, and transported to melanosomes as a mature 75-kD protein (Vijayasradhi et al., 1991). To identify the signals for intracellular sorting and targeting of gp75, we constructed chimeric proteins consisting of the extracellular domain of the T lymphocyte surface glycoprotein CD8, transmembrane (TM) domains of CD8 or gp75, and the cytoplasmic tail (Cyt) of gp75, and studied their cellular localization. Our data showed that the presence of the gp75 cytoplasmic tail was sufficient to cause intracellular retention of the chimeric CD8 molecules. Analysis of carboxyl-terminal deletion mutants of gp75 and chimeric CD8/gp75 showed that a sequence of six amino acids within the cytoplasmic tail of gp75 was necessary for sorting gp75 along the endocytic pathway in fibroblast transfectants. This peptide sequence is conserved between mouse and human gp75 and tyrosinase, and residues within this sequence are also conserved among other melanocyte-specific proteins known to be involved in regulation of melanin pigmentation.

Materials and Methods

Cells and Antibodies

Human melanoma cells SK-MEL-19, SK-MEL-23 clone 22a, and mouse L cell fibroblasts were cultured as described earlier (Bouchard et al., 1989; Vijayasradhi et al., 1991). Mouse hybridoma secreting anti-human CD8 mAb OKT-8 was obtained from American Type Culture Collection (Rockville, MD).

Purified mouse anti-human gp75 mAb TA99 IgG used for this study was described earlier (Vijayasradhi et al., 1990). Undiluted tissue culture supernatant of hybridoma OKT-8 collected after 48–72 h was used for staining CD8. Anti-mouse lysosomal membrane glycoprotein LAMP-1 antibody 1D4B (Chen et al., 1985) was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA).

Cloning and Expression of Full-length gp75 cDNA

A full-length 2.8-kb EcoRI fragment was isolated from a human melanoma cDNA library, and subcloned into the unique EcoRI site of eukaryotic expression vectors pCEXV3 (Bouchard et al., 1989) or pSVK3.1 (a derivative of vector pSVK3 obtained by deletion of the SacI fragment within the multiple cloning site), or SmaI site of pSVK3 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) after a fill-in reaction with Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA). The orientation of the cloned insert was determined by restriction analysis, and confirmed by dideoxy chain termination sequencing method (Sequenase kit, United States Biochemical Corp., Cleveland, OH) using an oligo-

nucleotide primer complementary to the vector sequences upstream of the cloning site.

Mouse L cell fibroblasts were transfected with plasmid containing gp75 cDNA and pSV2neo. Transfected clones were isolated by selecting for growth in the antibiotic G418 (1 mg/ml; GIBCO BRL, Gaithersburg, MD), and screened for gp75 expression by immunofluorescence staining with the mAb TA99 (Vijayasradhi et al., 1991).

Construction of CD8/gp75 Chimeric cDNA Expression Plasmids

The plasmid EBO-pCD-Leu2 containing human CD8- α cDNA was obtained from American Type Culture Collection (Margolske et al., 1988). The 2.3-kb BamHI fragment from this plasmid was isolated, made blunt-ended with Klenow fragment, and cloned into the SmaI site of the expression vector pSVK3. The orientation of the cDNA insert in the recombinant plasmids in *Escherichia coli* DH5- α was analyzed by appropriate restriction enzyme digestions, and confirmed by DNA sequencing.

Chimeric cDNAs encoding fusion proteins CD8/gp75(TM+Cyt), and CD8/gp75(Cyt) were constructed by the following methods. First, appropriate restriction sites at or near the TM/Cyt junction of CD8, and luminal/TM and TM/Cyt junctions of gp75 were generated by site-directed mutagenesis (Kunkel et al., 1987) using Mutagene kit (Bio-Rad Laboratories, Hercules, CA). Specifically, a mutant gp75 plasmid pSVgp75RV was generated by introducing an EcoRV restriction site at nucleotide 1560 (luminal/TM junction) of gp75 cDNA in plasmid pSVK3 using the mutagenic oligonucleotide 5'-TACTGCTATGGCAATGATATCAGGTACACTA-3' (mutations introduced are shown in bold). This resulted in the conversion of glutamic acid at position 477 (amino acids numbered starting with the methionine coded by the initiation codon) to aspartic acid. Mutant plasmids pSVgp75H and pSVleu2H were generated by introducing a HindIII restriction site in gp75 cDNA at nucleotide 1627, (gp75 TM/Cyt junction), and at nucleotide 706 (CD8 TM/Cyt junction) in CD8 cDNA using the mutagenic oligonucleotides 5'-GCGTCTGGCAGCAAGCTTATAAGAAGCAGT-3' and 5'-GTCTTCGGTTCCTAAGCTTGCAGTAAAGGGT-3', respectively. This resulted in conversion of leucine at position 500 to lysine, and isoleucine at 501 to leucine in gp75; and asparagine at 207 to lysine, and histidine at 208 to proline in CD8. Mutants were first identified by appropriate restriction enzyme digestion and confirmed by sequencing the relevant regions of the plasmids using Sequenase sequencing kit. Transient expression in mouse fibroblasts and immunofluorescence analyses with mAbs TA99 (anti-gp75) and OKT-8 (anti-human CD8) showed that intracellular staining of mutant proteins was identical to the distribution of wild-type counterparts, i.e., punctate cytoplasmic staining of gp75 and cell-surface expression of CD8.

Plasmid pSVgp75RV was digested with EcoRV and XbaI to produce an ~1.2-kb fragment containing the TM+Cyt sequence and 3' untranslated sequence of gp75 cDNA, including part of the multiple cloning site sequences of the vector; plasmid pSVleu2H was digested with EcoRV and XbaI and the large ~4-kb plasmid DNA fragment lacking TM and Cyt sequences of CD8 cDNA was isolated. The 1.2-kb EcoRV-XbaI gp75 fragment was ligated with the large EcoRV-XbaI pSVleu2H fragment to generate a plasmid construct encoding the fusion protein CD8/gp75(TM+Cyt). Similarly, an ~1-kb HindIII-XbaI gp75 cDNA fragment (containing gp75 Cyt and 3' untranslated sequences), and an ~4-kb HindIII-XbaI CD8 cDNA plasmid fragment (lacking the cytoplasmic tail sequences of CD8) were isolated, respectively, from plasmids pSVgp75H and pSVleu2H, and ligated to generate the fusion protein CD8/gp75(Cyt). Regions of the plasmids at the CD8/gp75 junctions were sequenced from at least two independent clones to confirm the restoration of the reading frame. Large-scale plasmid preparations (QIAGEN, Inc., Chatsworth, CA) were further verified by restriction enzyme digestions for the presence of enzyme sites unique to gp75 and CD8 at appropriate regions in the chimeric plasmids.

Generation of gp75 Carboxyl-terminal Deletion Mutants

pSVK3.1gp75 was used to generate carboxyl-terminal deletion mutants. The restriction enzyme site BglII at nucleotide 2000 of gp75 cDNA is a unique site within the plasmid pSVK3.1. Plasmid pSVK3.1gp75 (10 μ g) was linearized by digestion with 40 U of BglII in a 50- μ l reaction for 3 h at 37°C. Linearized ~6.7-kb DNA was then digested for 3–4 min with Bal 31 nuclease (1 U enzyme/ μ g DNA) in a 50- μ l reaction. Digested DNA was immediately extracted with phenol/chloroform to inactivate and remove the nuclease, and the ends were filled in by Klenow fragment of DNA

polymerase I to increase the population of blunt-ended molecules (Sambrook et al., 1989). Klenow fragment was inactivated by heating at 75°C for 10 min, and a suppressible reading frame termination linker containing restriction site *NheI*, 5'-CTAGCTAGCTAG-3' (Pharmacia LKB Biotechnology Inc.), was ligated to the blunt-ended, truncated pSVK3.1gp75 DNA molecules with 1 U of T4 DNA ligase in a 20- μ l reaction for 3 h at room temperature. The ligation mixture was used to transform *E. coli* strain DH5- α . Ampicillin-resistant bacterial colonies were analyzed by agarose gel slot lysis method for the presence of plasmid DNA of appropriate size. Plasmid DNA from 15 transformants was isolated, analyzed by restriction enzyme digestion, and partially sequenced to determine the number of bases deleted from the carboxyl terminus, and to confirm the addition of termination linker.

Transfection and Transient Expression

A transient transfection method was developed and optimized to study the intracellular distribution of gp75 expressed by mutant constructs. Briefly, $2-4 \times 10^4$ SK-MEL-23 clone 22a melanoma cells and mouse L cells fibroblasts were plated in 8-well chamber slides (Lab-Tek, Naperville, IL). The cells were transfected with plasmid DNA by calcium phosphate precipitate method for 16–24 h, and then allowed to accumulate the expressed protein for 12–48 h.

Immunofluorescence Microscopy

Cells on the 8-well glass slides were fixed with formaldehyde, followed by methanol, and stained with gp75-specific mouse mAb TA99 or OKT-8 followed by FITC-conjugated anti-mouse IgG. Cells were examined under fluorescence microscope (Optiphot; Nikon Inc., Instrument Group, Melville, NY) and photographed using Ektachrome film (Eastman Kodak Co., Rochester, NY).

Immunoelectronmicroscopy

mAb TA99 directly conjugated to 10-nm gold particles was used for localization of gp75 by immunoelectron microscopy. Colloidal gold was prepared as described (Smit and Todd, 1986) and mAb TA99-gold conjugate was prepared according to Alexander et al. (1985).

Human melanoma SK-MEL-19 cells were fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, infused with 2.3 M sucrose in PBS, and the cell pellet was then frozen in liquid nitrogen. Ultrathin sections were cut and collected on Formvar-carbon coated nickel grids. The sections on the grids were incubated in 0.5% BSA in PBS to block non-specific protein binding sites, and then stained with mAb TA99 conjugated to 10 nm gold particles. Washing and staining of the sections were performed according to Griffiths et al. (1983). Sections were observed on an electron microscope (100CX; JEOL USA Analytical Instruments, Cranford, NJ).

Metabolic Pulse-Chase Labeling, Endoglycosidase H (Endo H) Digestion, Immunoprecipitation, and SDS-PAGE Analysis

Transfected fibroblasts grown in 25-cm² tissue culture flasks were labeled for 30 min, and for pulse-chase analysis, SK-MEL-19 melanoma cells and transfected fibroblasts were labeled 5 min with 100 μ Ci of [³⁵S] methionine (EXPRE³⁵S³⁵S protein labeling mix; New England Nuclear, Boston, MA), washed twice, and chased in normal growth medium containing cold methionine for indicated periods. Cells were harvested, washed twice with ice-cold PBS, and lysed in 150–200 μ l lysis buffer (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 2 μ g/ml aprotinin, 0.7 μ g/ml pepstatin, 0.5 mg/ml leupeptin, and 0.2 mM PMSF). The lysates were cleared by centrifugation at 15,000 *g* for 10 min at 4°C, and stored at –20°C. Incorporation of radioactivity into proteins was determined by precipitation with TCA. Briefly, 1–2 μ l of cell lysate was spotted on glass fiber filters (Whatman Inc., Clifton, NJ) on multifiltration apparatus (Millipore Corp., Bedford, MA), and each filter was washed with 4 \times 4 ml ice-cold 10% TCA, dehydrated with absolute ethanol, and air-dried. Protein-bound radioactivity was measured in Liscisint (National Diagnostics Inc., Manville, NJ) in a scintillation counter.

Appropriate volumes of cell lysates containing $1-2 \times 10^6$ TCA-insoluble cpm were made up to 0.2 ml with lysis buffer, and incubated with mAb TA99 (300 μ g/ml diluted in gamma globulin free fetal bovine serum) for 1–2 h at 4°C. At the end of incubation with the primary antibody, a 40–50-

μ l suspension of rabbit anti-mouse IgG coupled to protein A Sepharose (10% wt/vol) was added and incubated for 2–4 h or overnight at 4°C. Immunoprecipitates were washed eight times with 10 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 1% NP-40 (TNEN), four times with one-tenth strength TNEN containing 0.5 M NaCl, and finally with distilled water (Vijayasaradhi et al., 1991).

For Endo H digestion, the immunoprecipitates were dissociated by suspending in 20 μ l 0.2% SDS, heated for 5 min at 100°C, and diluted with 20 μ l 0.1 M sodium citrate buffer, pH 5.5, Endo H, 50 mU/ml; digestion was carried out at 37°C for 18–24 h. The reaction mixture was layered over with 50 μ l toluene. Control tubes were treated similarly, except that equal volume of 0.05 M sodium citrate buffer, pH 5.5, was added instead of Endo H.

Proteins were analyzed by 9% SDS-PAGE. Radioactive protein bands were visualized by fluorography. Prestained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) are lysozyme, 15,000; β -lactoglobulin, 18,000; α -chymotrypsinogen, 25,000; ovalbumin, 42,000; BSA, 66,000; phosphorylase B, 104,000; myosin (H-chain), 199,000.

Results

Human gp75 cDNA encodes a 537-amino acid-long polypeptide consisting of amino-terminal signal peptide sequence, a long amino-terminal domain, a TM region, and a 36-amino acid-long carboxyl-terminal domain (Fig. 1). Light and electron microscopic localization studies showed that in melanocytic cells, gp75 is localized to juxtannuclear membranes and melanosomes (Fig. 2). Very little, or no expression of gp75 could be detected on the cell surface of human melanoma cells using sensitive techniques, such as mixed hemadsorption assay and cell-surface labeling with radioactive iodine (data not shown). To investigate the protein structural requirements for intracellular sorting and tar-

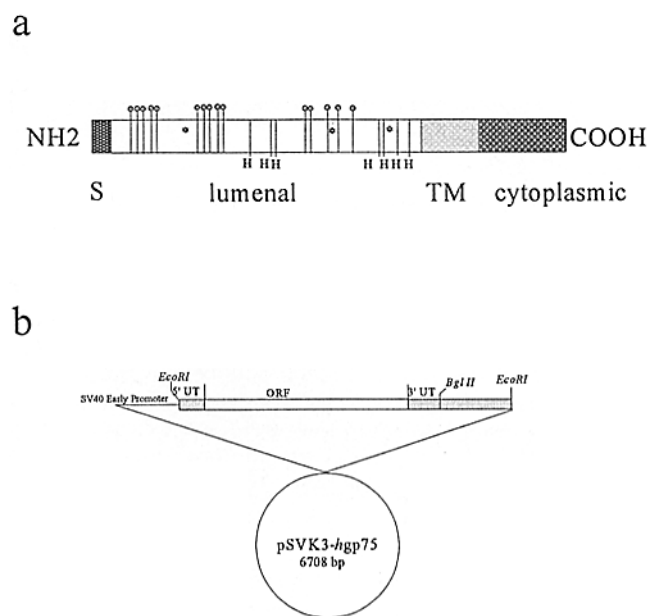


Figure 1. Schematic diagrams of (a) gp75 showing the protein domains and the structural features conserved among members of the tyrosinase-related family, and (b) expression vector containing gp75 cDNA with the restriction sites relevant to this study. (a) S, signal peptide; TM, transmembrane region. Vertical bars projecting up with a dot, conserved cysteine residues. Vertical bars projecting down, conserved histidines (required for binding copper). Asterisks, conserved Asn-linked glycosylation acceptor sites. (b) ORF, open reading frame; UT, untranslated region. Bars and circle not drawn to scale.

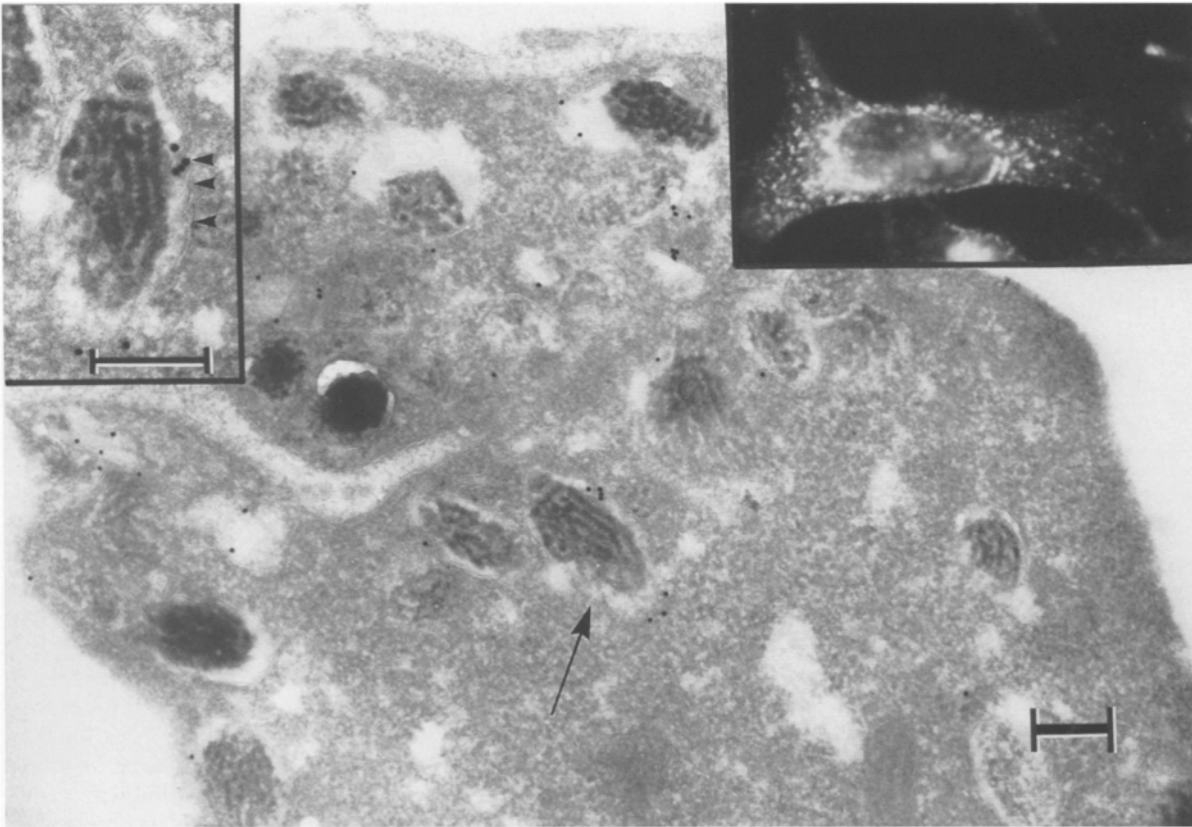


Figure 2. Subcellular distribution of gp75. Immunoelectron microscopic analysis of human melanoma cells SK-MEL-19 using 10-nm gold particles conjugated to mAb TA99. (Arrows) gp75 localized to the melanosomal membrane. (Left inset) A high magnification view of the melanosome marked by arrow shows the contiguous melanosomal membrane (arrowheads) to which gp75 is localized. (Right inset) Immunofluorescent staining of SK-MEL-19 cell with mAb TA99 showing bright juxtannuclear staining and the distribution of gp75 in cytoplasmic vesicles. Bars, 0.25 μm .

getting of gp75 to melanosomes, we constructed protein chimeras, generated deletion mutants, and studied the subcellular distribution of mutant and chimeric proteins transiently expressed in fibroblasts.

Because we hypothesized that the carboxyl tail of gp75 contained a sorting signal, we first had to investigate the membrane orientation of gp75. Postnuclear membranes from metabolically labeled melanoma cells were digested with proteinase K or trypsin before detergent solubilization and immunoprecipitation with mAb TA99. SDS-PAGE analysis showed a broad band of 65–70 kD, ~ 5 kD smaller than the undigested gp75 protein (data not shown). This is consistent with a membrane orientation of gp75 in which the bulk of the protein, with its glycosylated amino-terminal domain, is inside the melanosomal lumen (inaccessible to the proteases) and the ~ 5 -kD short carboxyl tail is located outside, facing the cytosol.

Next, we established that biosynthesis and processing of gp75 is similar in fibroblasts and melanocytic cells. Mouse L cell fibroblasts were cotransfected with gp75 cDNA and pSV2neo plasmid, and G418-resistant clones that stained positive with mAb TA99 were isolated. Immunoprecipitation analysis of the cell lysates of transfectants metabolically labeled with [^{35}S]methionine showed that mAb TA99 precipitated a broad 72–75-kD band identical to melanoma gp75. mAb TA99 recognizes conformational epitopes

on glycosylated gp75 (Vijayasaradhi et al., 1991), so the ability of mAb TA99 to precipitate gp75 from the transfectants demonstrated that the protein synthesized in mouse fibroblasts was structurally similar to the human melanoma gp75.

We compared the time-course of intracellular movement of gp75 in transfected fibroblasts and human melanoma cells. Fig. 3 shows pulse-chase analyses of posttranslational processing of gp75 in SK-MEL-19 melanoma cells and transfected fibroblast clone. At the end of 5 min pulse labeling, newly synthesized gp75 appeared as a major 69-kD band (and a minor 67-kD band) in melanoma cells, and a single 69-kD band in fibroblasts. Digestion with Endo H, which removes high mannose Asn-linked oligosaccharides, produced a 55-kD core polypeptide band showing that the 69- and 67-kD bands represented the ER and *cis*-Golgi forms of gp75 with high mannose oligosaccharide chains. With increasing periods of chase, the 55-kD band produced by Endo H digestion became less prominent with concomitant appearance of bands having reduced electrophoretic mobility. After 2 h chase, in both melanoma cells and transfected fibroblasts, Endo H digestion produced only the higher mol wt band(s) but not the 55-kD band. These data showed that processing through the medial- and *trans*-Golgi and maturation of gp75 in fibroblasts occurred at a rate similar to that in melanocytic cells.

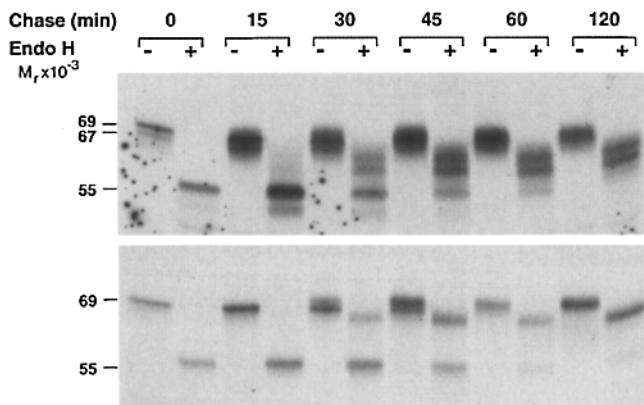


Figure 3. Pulse-chase analysis of oligosaccharide processing of gp75 in human melanoma cells SK-MEL-19 (*upper panel*) and clone 7-transfected fibroblasts (*lower panel*). Cells were pulse labeled with [³⁵S]methionine for 5 min and chased for increasing lengths of indicated time. gp75 protein was immunoprecipitated and analyzed by SDS-PAGE with (+) or without (-) Endo H digestion. Numbers on the left are apparent molecular masses (*kD*) of the protein bands.

Colocalization of gp75 and Lysosomal Membrane Proteins in Transfected Fibroblasts

Indirect immunofluorescence staining of transfected fibroblasts with mAb TA99 showed intracellular juxtannuclear or perinuclear staining, and a punctate cytoplasmic staining similar to the pattern observed in human melanocytic cells. In melanocytic cells, the punctate cytoplasmic staining represented gp75 targeted to melanosomes and their precursors. Double immunofluorescence staining of fibroblast transfectants with mAb TA99 and anti-LAMP-1 antibody 1D4B showed that almost all intracellular structures that stained positive for melanosomal gp75 also stained with the antibody to LAMP-1 (Fig. 4). The targeting of gp75 to LAMP-1-positive structures showed that, in fibroblasts, gp75 was sorted along the endosomal/lysosomal pathways. This is consistent with the observation that

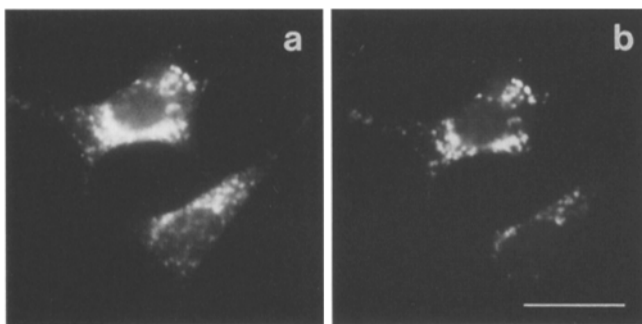


Figure 4. Colocalization of gp75 and LAMP-1 in transfected fibroblasts. Cells fixed in 2% paraformaldehyde and permeabilized with cold methanol were first incubated with rat anti-mouse LAMP-1 antibody (mAb 1D4B), followed by TRITC-conjugated rabbit anti-rat IgG, and finally with FITC-conjugated anti-gp75 antibody (mAb TA99). *a*, gp75 and *b*, LAMP-1. Cells were photographed at the same plane of focus using Optiphot microscope with appropriate optical filters and a barrier filter. Bar, 30 μ m.

in 3T3-fibroblast transfectants, mouse gp75 was localized to the LAMP-1- and β -glucuronidase-positive endosomal/lysosomal structures (Winder et al., 1993). These data showed that signals responsible for sorting and targeting of gp75 to intracellular vesicles (melanosomes in melanocytic cells) are also functional in fibroblasts.

Cytoplasmic Domain of gp75 Influences the Cellular Localization of CD8/gp75 Chimeras

To localize the signals responsible for intracellular sorting and targeting of gp75 to lysosomes, we constructed chimeric proteins consisting of the extracellular domain of the T lymphocyte surface protein CD8 and the TM and cytoplasmic domains of gp75 (CD8/gp75 TM+Cyt), and the extracellular and TM domains of CD8 and the cytoplasmic domain of gp75 (CD8/gp75 cyt) (Fig. 5). Cellular localization of wild-type CD8, CD8 protein lacking the cytoplasmic tail (tailless CD8), and the CD8/gp75 chimeric proteins expressed in fibroblasts was studied by immunofluorescence staining with mAb OKT-8, which recognizes epitope(s) on the extracellular domain of CD8 molecule.

Membrane-permeabilized fibroblasts expressing full-length wild-type CD8 showed a pattern of staining that demarcated the cellular margins, with a diffuse staining over the cell body and occasional patches of intense staining near the margins (Fig. 6 *a*). Staining of only the cellular margins was observed in cells expressing the mutant CD8 lacking the cytoplasmic tail (Fig. 6 *c*). In these cells, identical staining pattern was observed with or without membrane permeabilization. These data are consistent with localization of CD8 protein on the cell surface with its amino-terminal domain facing outside. Cell-surface expression of the tailless CD8 protein in transfected fibroblasts showed that the cytoplasmic tail of CD8 had no influence on the cellular localization of CD8 protein, or the ability of mAb OKT-8 to recognize epitopes on the extracellular amino-terminal domain of CD8.

In membrane-permeabilized transfectants expressing the chimeric CD8/gp75 TM+Cyt and CD8/gp75 Cyt proteins, the anti-CD8 antibody staining was localized to the

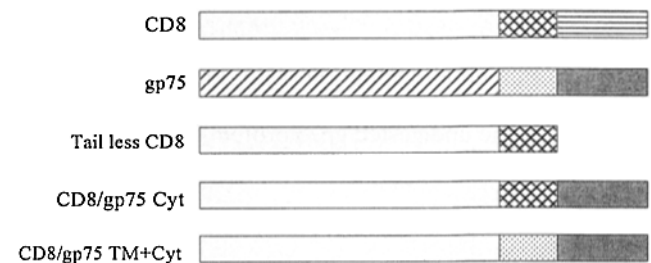
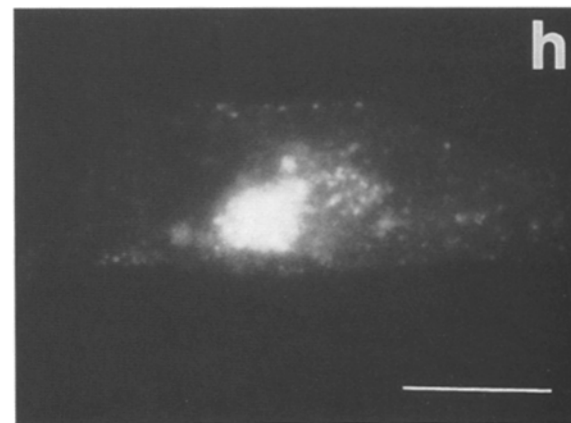
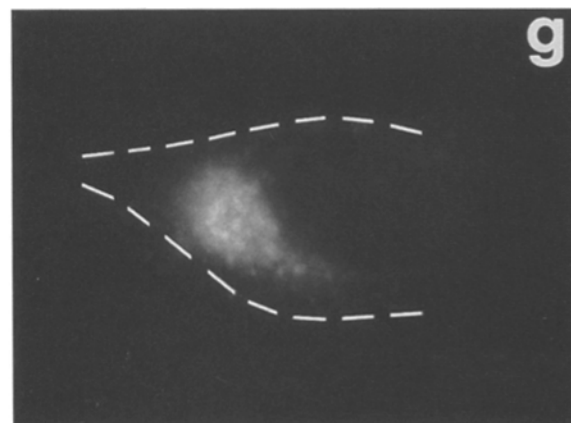
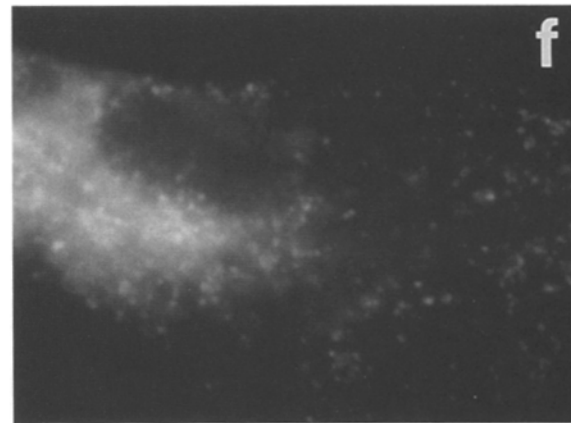
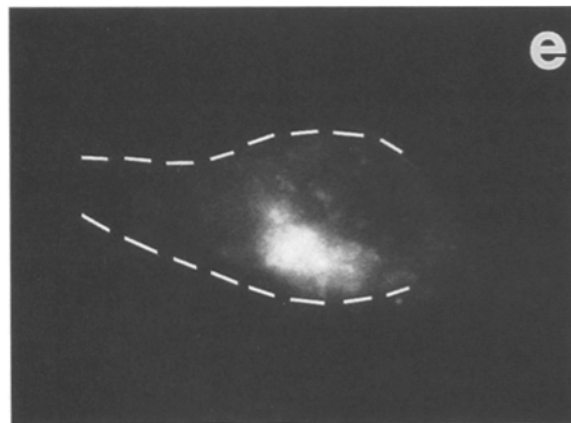
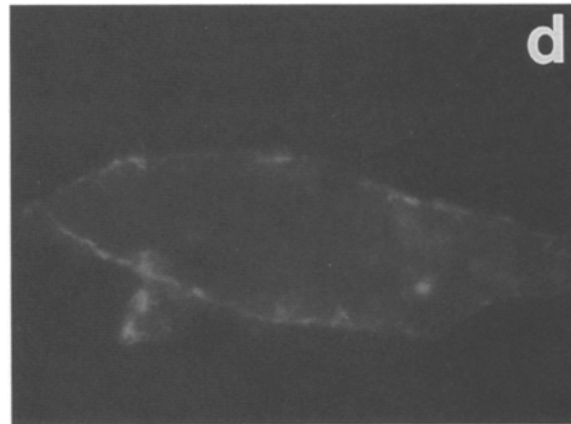
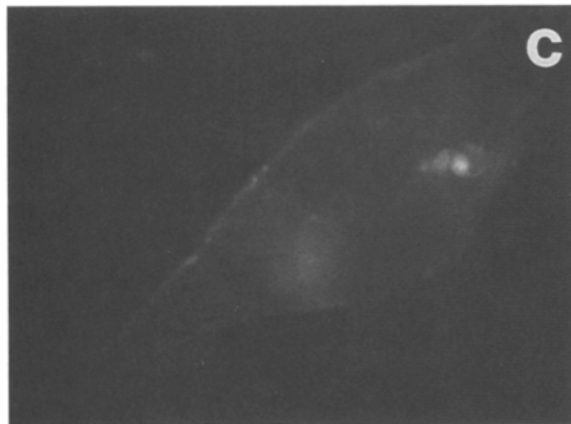
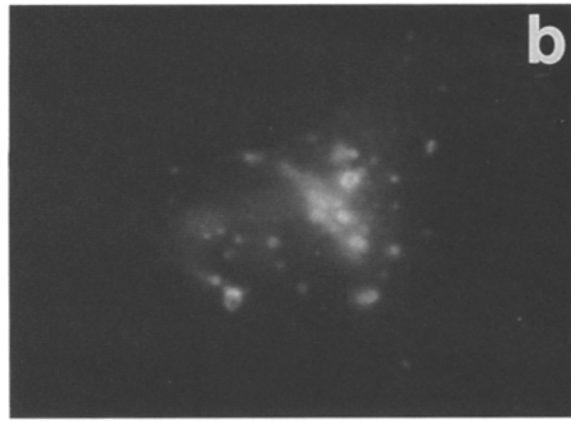
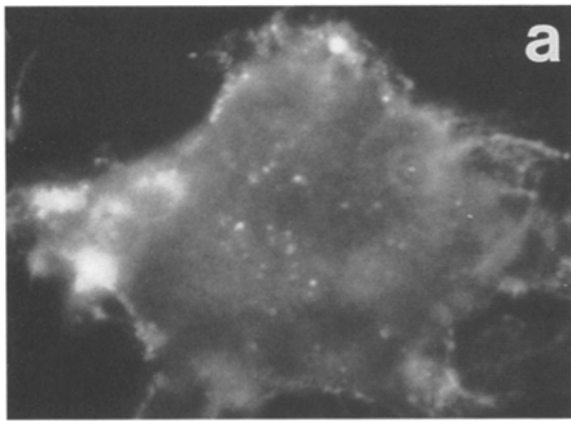


Figure 5. Schematic diagram of wild-type CD8 and gp75 proteins, tailless CD8 mutant, and CD8/gp75 chimeras. CD8 amino-terminal domain, transmembrane region, and the cytoplasmic tail are shown as an open bar, a box with cross hatches, and a box with horizontal lines, respectively. For gp75, the amino-terminal region is shown as hatched bar, transmembrane region as a stippled box, and the cytoplasmic tail as a solid box. CD8 chimeras containing either gp75 cytoplasmic tail alone, or the gp75 transmembrane region and cytoplasmic tail were constructed as described in Materials and Methods.



juxtannuclear region. There was very little, or no staining of other cytoplasmic structures or the plasma membrane (Fig. 6, *e* and *g*). The pattern of staining suggested localization of the chimeric proteins in the Golgi region and possibly other organelles such as late endosomes and lysosomes present in the Golgi region. The exclusive localization of the chimeric proteins in the Golgi region, and absence of these proteins in peripheral cytoplasmic organelles could be the result of a rapid degradation of the CD8/gp75 chimeras in a proteolytic compartment. Because our studies on the cellular localization of the wild-type gp75 showed that the protein expressed in fibroblasts was localized to LAMP-1-positive vesicles, we tested the possibility that CD8/gp75 chimeras were transported to endosomes/lysosomes where the extracellular domain of CD8 was rapidly degraded by proteases. Mouse L cell fibroblasts were transfected with cDNAs encoding CD8/gp75 chimeras, and the transiently expressed proteins were allowed to accumulate for 24 to 72 h in the presence or absence of the serine protease inhibitor, leupeptin. Cells were fixed and permeabilized at 24, 48, and 72 h, and the cellular distribution of the chimeras was analyzed by immunofluorescence staining with mAb OKT-8. In cells expressing CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeric proteins in the absence of leupeptin, the anti-CD8 antibody staining was localized to the juxtannuclear region (Fig. 6, *e* and *g*), and remained localized to that region even 72 h after transfection (data not shown). When leupeptin was added in the culture medium of transfectants, staining of organelles distributed throughout the cytoplasm, in addition to the juxtannuclear staining, could be seen by 24 h (data not shown) and became prominent by 48 h (Fig. 6, *f* and *h*). Both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras showed identical staining pattern. Leupeptin did not alter the cell-surface localization of the tailless CD8 protein (Fig. 6 *d*). These results allowed us to draw the following conclusions on the sorting of CD8/gp75 chimeras: (*a*) In fibroblasts, both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras were localized in intracellular compartments and failed to accumulate at the cell surface. (*b*) Both chimeras were sorted to a cellular compartment where the extracellular domain of CD8 was rapidly degraded in the absence of protease inhibitors. (*c*) The cytoplasmic tail of gp75, the common domain in both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras, was necessary for intracellular sorting of the CD8 chimeras to a protease-rich compartment, consistent with endosome/lysosome.

Carboxyl-terminal 27 Amino Acids Are Necessary for Intracellular Sorting of gp75

To delineate further the region and amino acid sequences within the gp75 cytoplasmic domain that determine sort-

ing along the endocytic pathway, we generated carboxyl-terminal deletion mutants of gp75. Deletions of varying lengths were generated by Bal I nuclease digestion of linearized gp75 plasmid followed by ligation of a multiple reading frame termination linker.

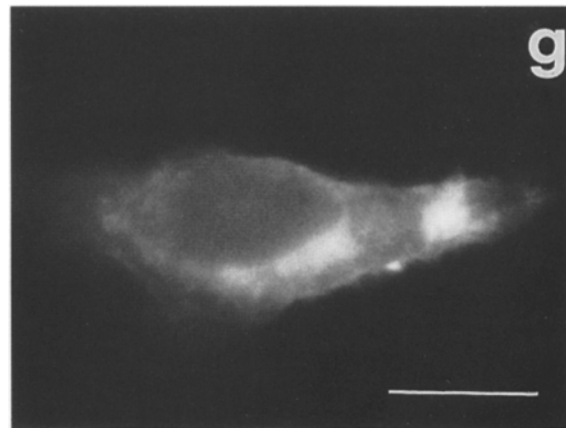
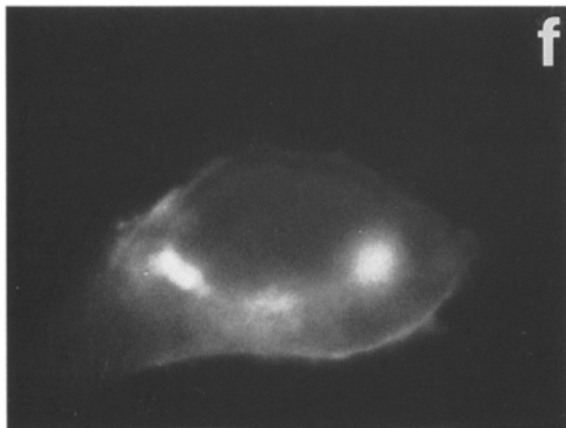
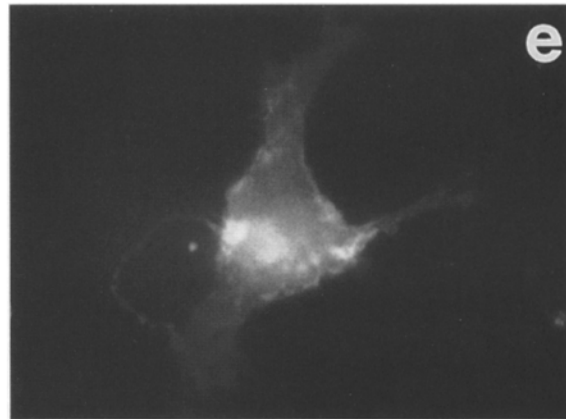
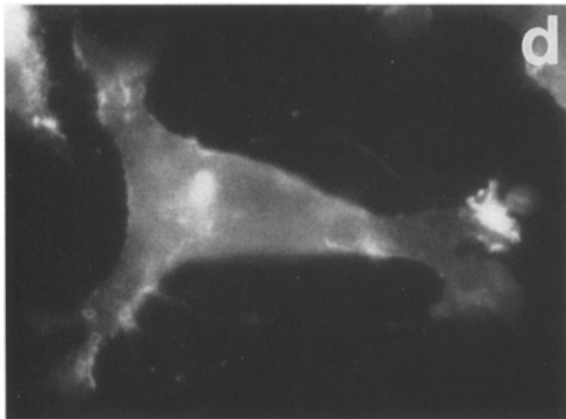
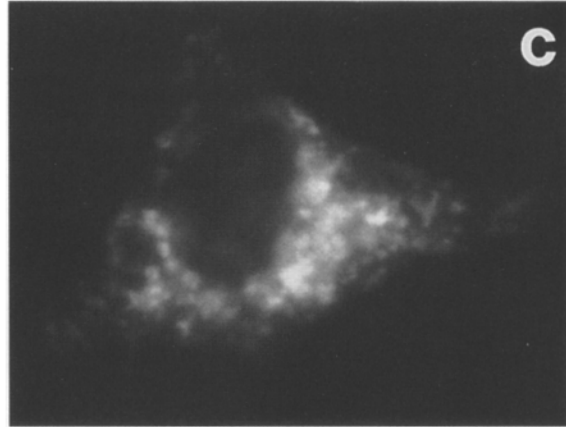
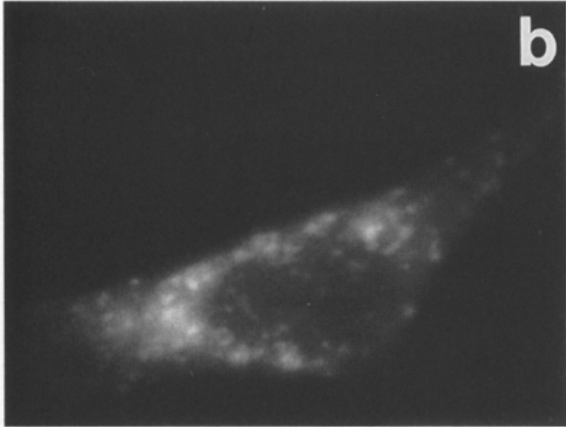
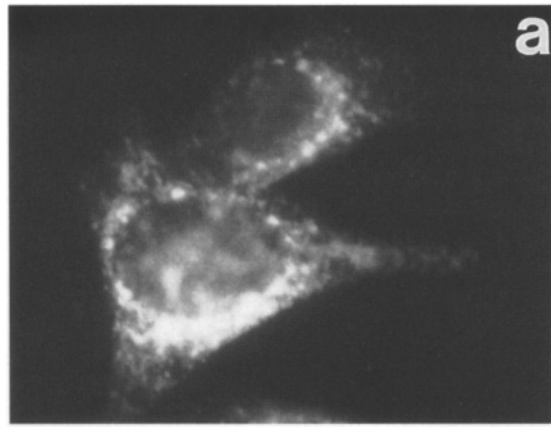
Mouse L cell fibroblasts and a nonpigmented, melanosome-positive and gp75-negative melanoma cell line (SK-MEL-23 clone 22a) were transfected with the carboxyl-terminal deletion mutants of gp75, and the cellular distribution of transiently expressed protein was studied by immunofluorescent staining with the mAb TA99 (Figs. 7 and 8). Transfectants expressing mutant gp75 proteins with deletion of 14 or 20 carboxyl-terminal amino acid residues showed a patchy juxtannuclear staining and punctate cytoplasmic staining (Figs. 7 and 8, *b* and *c*). This pattern of staining was similar to the staining of the wild-type full-length gp75 in transfectants, and also to the distribution of gp75 constitutively expressed in melanoma cells.

Expression of deletion mutants of gp75 lacking the terminal 27 or 34 amino acids in melanoma cells SK-MEL-23 clone 22a and fibroblasts produced a staining pattern that demarcated the cellular boundaries of membrane-permeabilized cells, and a patchy to diffuse intracellular staining (Fig. 7, *f* and *g*; Fig. 8, *d* and *e*), similar to the staining observed in fibroblasts expressing the full-length CD8 protein. Anti-gp75 staining could be seen without membrane permeabilization (Fig. 7, *d* and *e*). These results showed that deletion of up to 20 carboxyl-terminal amino acid residues (Δ C20 construct) did not affect the sorting and intracellular retention of gp75 in melanoma cells. Deletion of an additional seven amino acids, i.e., 27 carboxyl-terminal amino acids (Δ C27 construct), resulted in failure of gp75 to be sorted efficiently for intracellular retention, and the protein appeared on the cell surface. These data demonstrated that amino acid residues between carboxyl-terminal positions 20 and 27 were responsible for intracellular sorting, and suggested that these residues are involved in targeting of gp75 to melanosomes.

Amino Acid Residues between 511 and 517 within the Cytoplasmic Tail of gp75 Constitute a Signal for Retention and Intracellular Sorting of gp75

Carboxyl termini of the deletion mutant Δ C20, which was sorted for retention in intracellular compartments, and the deletion mutant Δ C27, which was expressed on the cell surface, delineated the limits of a 7-amino acid sequence, *asn-gln-pro-leu-leu-thr-asp* (NQPLLED), that was necessary for the intracellular sorting and targeting of gp75 in both melanocytic cells and fibroblasts (Fig. 9). Substitution of carboxyl-terminal residues of truncated gp75 molecules with amino acid residues encoded by the termination linker did not affect the cellular localization determined

Figure 6. Immunofluorescence microscopy of L cells transfected with wild-type CD8, tailless CD8, and CD8/gp75 chimeric plasmid constructs. Transfected cells expressing wild-type CD8 (*a*), wild-type gp75 (*b*), tailless CD8 (*c* and *d*), CD8/gp75 TM+Cyt (*e* and *f*) or CD8/gp75 Cyt (*g* and *h*) were fixed in 2% paraformaldehyde 48 h after transfection. Cells were stained with anti-CD8 mAb OKT-8 (*a*, *c*–*h*) or rabbit anti-human gp75 antibody (*b*). Cells in *a*, *b*, and *e*–*h* were stained after membrane permeabilization, and cells in *c* and *d* were stained without membrane permeabilization. In the absence of the protease inhibitor leupeptin (*e* and *g*), both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras were localized exclusively to the juxtannuclear Golgi region. (*e* and *g*) White dashes indicate the cellular margins. In the presence of leupeptin (*f* and *h*), staining of distinct cytoplasmic organelles could be seen. Leupeptin had no effect on the cell-surface expression of the tailless CD8 protein (*d*). Bar, 16 μ m.



by this sorting signal, as demonstrated by different patterns of distribution of $\Delta C14$, $\Delta C27$, and $\Delta C34$ (Figs. 7 and 8, *b, d*, and *e*) mutants containing identical carboxyl-terminal linker residues. These mutants also showed the lack of involvement of additional carboxyl-terminal residues, specifically the glutamine residue at position 518, and the tyrosine residue at position 526, in sorting gp75.

To test whether the NQPLLED sequence was responsible for intracellular retention of the CD8/gp75 chimeras, we made mutant CD8/gp75 chimeras with the gp75 cytoplasmic tail lacking 20 or 27 carboxyl-terminal amino acids. This was accomplished by introducing a translation stop codon at positions 512 (*gln*) or 518 (*gln*) within the gp75 cytoplasmic tail of the CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras. As shown earlier, fibroblasts transfected with the wild-type chimeras stained with anti-CD8 antibody showed characteristic juxtannuclear staining in the absence of the protease inhibitor, leupeptin, and punctate cytoplasmic staining in the presence of leupeptin. Expression of mutant chimeras with the gp75 cytoplasmic tail lacking the carboxyl-terminal 20 amino acids produced a staining pattern identical to the wild-type chimeras (Fig. 10, *a* and *d*). In transfectants expressing the mutant chimeras lacking the 27 carboxyl-terminal amino acids, a staining pattern identical to the staining of the wild-type full-length and tailless CD8 proteins was observed (Fig. 10, *c* and *f*). This staining could be detected without membrane permeabilization, and without the addition of leupeptin during the transient expression, demonstrating the cell-surface localization of the chimeric CD8 protein. These data showed that the peptide sequence NQPLLED within the cytoplasmic domain of gp75 was also necessary for the intracellular sorting and targeting of the CD8/gp75 chimeras to endosomes/lysosomes in fibroblasts.

QPLLED Is Conserved among Melanosomal Membrane Proteins

The hexapeptide sequence QPLLED, consisting of a polar uncharged residue followed by three nonpolar hydrophobic residues, is conserved between both mouse and human gp75 (Fig. 11). Carboxyl-terminal deletion analysis studies showed that the cytoplasmic tail was also necessary for intracellular sorting of mouse gp75 (Xu, Y., S. Vijayasaradhi, and A. N. Houghton, manuscript in preparation). We examined the amino acid sequences of other melanosomal membrane proteins for the presence of similar intracellular sorting signals, including tyrosinase encoded by the *albino* locus, TRP-2 encoded by the *slaty* locus, Pmel 17 encoded by the *silver* locus, P-protein encoded by the *pink-eyed* (*p*) locus, and melanocyte stimulating hormone receptor encoded by the *extension* locus. A conserved pep-

tide sequence QPLLED is present at a similar position (22 amino acids from the TM domain) in the cytoplasmic tail of both human and mouse tyrosinases. In the carboxyl terminus of Pmel 17 protein, a sequence NSPLLSG is present 36 amino acids downstream from the putative TM domain. Recently, Zhou et al. (1994) have shown that the Pmel 17 is processed to a melanosomal matrix protein. In the human *p*-locus gene product (which is presumably a melanosomal membrane protein with 12 transmembrane domains), a sequence NTPLLR, and in mouse *p*-locus protein, TPLLWN, are present at similar positions within the amino-terminal region. It is important to note that the amino-terminal region of the *p*-locus protein has been predicted to face the cytoplasmic face of the melanosomal membrane (Rinchik et al., 1993). Presence of this conserved sorting signal in the amino-terminal region of the *p* protein supports both its melanosomal localization, and is consistent with the cytoplasmic orientation of this conserved region in gp75, tyrosinase and Pmel 17. In addition to the residues within the hexapeptide sequence, a glutamic acid located 3–4 residues amino-terminal to the dileucine motif is also conserved among all these melanosomal proteins. No sequences of significant homology to QPLLED were found in TRP-2, a melanogenic enzyme localized to the melanosome (Fig. 11) and the *e* locus product, a receptor for melanocyte stimulating hormone (sequence not shown).

Discussion

The biogenesis of melanosomes requires intracellular sorting and targeting of melanosomal membrane proteins to appropriate precursor vesicles. This family of glycoproteins include tyrosinase (the *albino* locus product), gp75 (*brown* locus), and Pmel 17 (*silver* locus product). In this study, we have investigated the signals responsible for intracellular sorting of the most abundant melanosomal glycoprotein, gp75. Chimeric CD8 proteins containing the carboxyl tail of gp75, with TM domain of either CD8 or gp75, were retained in the cell and failed to reach the cell surface. This demonstrated that the 36-amino acid carboxyl tail of gp75 was responsible for the intracellular sorting of the chimeric CD8 molecule. Intracellular staining was localized almost exclusively to the juxtannuclear Golgi region after membrane permeabilization. The apparent accumulation of the chimeric CD8 molecules in the Golgi region was not due to retardation of plasma membrane protein transport. When leupeptin was added to the cells after transfection, staining of cytoplasmic vesicles could be seen as early as 24 h, showing that signals in the gp75 carboxyl tail were sufficient to target the chimeric proteins to

Figure 7. Immunofluorescence microscopy of L cell fibroblasts transiently expressing wild-type gp75 (*a*) and carboxyl-terminal deletion mutants of gp75 $\Delta C14$ (*b*), $\Delta C20$ (*c*), $\Delta C27$ (*d* and *f*), or $\Delta C34$ (*e* and *g*). Transfected cells were allowed to express the proteins for 48 h, were fixed in 2% paraformaldehyde, and were stained with mAb TA99 followed by FITC-conjugated anti-mouse IgG with (*a-c, f*, and *g*) or without (*d* and *e*) prior membrane permeabilization. In membrane-permeabilized cells, $\Delta C14$ and $\Delta C20$ mutants (*b* and *c*) produced an intracellular staining pattern similar to the full-length wild-type gp75, whereas mutants $\Delta C27$ and $\Delta C34$ (*f* and *g*) showed a distinct staining of cellular margins and a weak intracellular staining. Cells in all panels are shown at identical magnification except in *d* and *e* in which cells are shown at a lower magnification. Bars, 16 μm (*a-c, f*, and *g*); 20 μm (*d* and *e*).

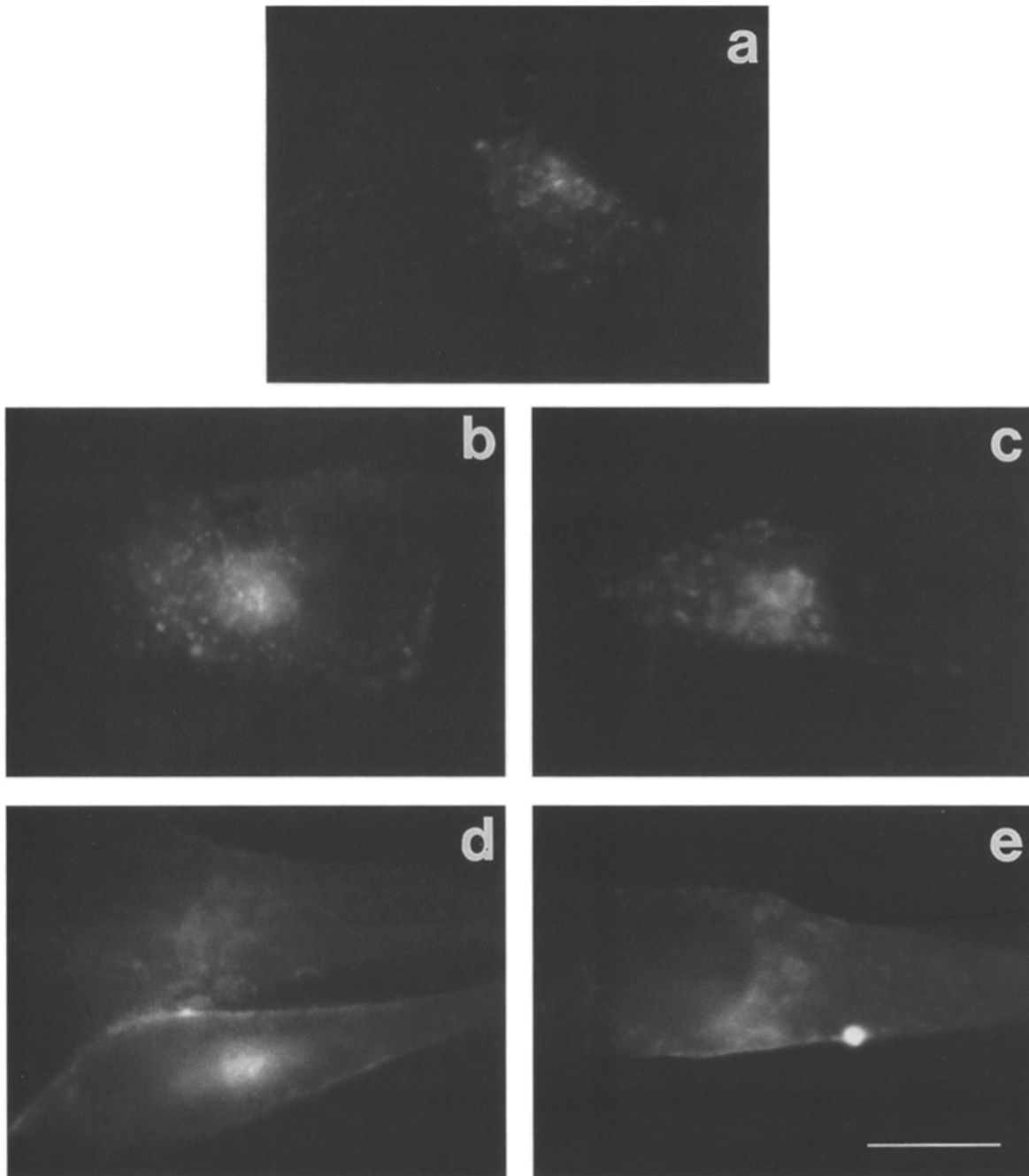


Figure 8. Immunofluorescence microscopy of human melanoma cells (clone 22a) transiently expressing wild-type gp75 (*a*) and carboxyl-terminal deletion mutants of gp75 Δ C14 (*b*), Δ C20 (*c*), Δ C27 (*d*), or Δ C34 (*e*). Transfected cells were allowed to express the proteins for 48 h, and were fixed in 2% paraformaldehyde, permeabilized, and stained with mAb TA99, followed by FITC-conjugated anti-mouse IgG. In membrane permeabilized cells, Δ C14 and Δ C20 mutants (*b* and *c*) produced an intracellular staining pattern similar to the full-length wild-type gp75, whereas mutants Δ C27 and Δ C34 (*d* and *e*) showed a distinct staining of cellular margins and a weak intracellular staining. Bar, 16 μ m.

the protease-rich vesicular components of the endosomal/lysosomal pathway. In this proteolytic compartment, the amino-terminal domain of CD8 was rapidly degraded, whereas the highly glycosylated luminal domain of gp75 was stable. Newly synthesized gp75 in fibroblast transfectants has an apparent half-life of 20–24 h, similar to the long half-life of gp75 in melanoma cells (Vijayasradhi et al., 1991; and Vijayasradhi, S., and A. N. Houghton, un-

published observations). Sequential carboxyl-terminal deletion mutants allowed us to identify the six-amino acid sequence, Asn-Gln-Pro-Leu-Leu-Thr, critical for the intracellular sorting of gp75 in melanocytic cells and the CD8 chimeras along the endocytic pathway.

Upon exit from the TGN, newly synthesized membrane proteins destined for intracellular retention and targeting to organelles are transported to late endosomes. In addi-

wt gp75 ⁵⁰²RARRSMDEAN**QPLLTDQYQCY**AEEYEKLQNPNSVV

Δ C14 RARRSMDEAN**QPLLTDQYQCY**⁵²³*ALAS*

Δ C20 RARRSMDEAN**QPLL**⁵¹⁷*TDS*

Δ C27 RARRSMDEA⁵¹⁰*ALAS*

Δ C34 ⁵⁰³*RALAS*

Figure 9. Amino acid sequences at the carboxyl termini of wild-type gp75 and deletion mutants. Sequences are shown beginning at the junction of membrane spanning region (TM) predicted from hydrophobicity analysis and the cytoplasmic tail. Amino acid residues are numbered from the amino terminus, beginning with the methionine residue encoded by the initiation codon. Numbers after ΔC represent the gp75 amino acid residues deleted from the carboxyl terminus. Amino acid residues required for the intracellular sorting of gp75 are shown in bold. gp75 terminal amino acids replaced by amino acids encoded the termination linker are shown in italics.

tion to newly synthesized proteins transported from the TGN, late endosomes also receive input from the plasma membrane and extracellular environment. Some cell-surface receptors internalized by endocytosis recycle between

early and late endosomes before returning to the plasma membrane or transported to lysosomes for degradation (Trowbridge et al., 1993). Biogenesis of distinct postendosomal compartments such as lysosomes (Peters and von Figura, 1994), transcytotic vesicles, synaptic vesicles (Kelly, 1993), major histocompatibility complex class II containing vesicles (Amigorena et al., 1994; Tulp et al., 1994), and, presumably, melanosomes, requires specific endosomal sorting of intracellular membrane proteins from proteins that recycle between early and late endosomes and plasma membrane.

Intracellular pathways of sorting and targeting of integral membrane proteins to the lysosome have been investigated extensively (Peters and von Figura, 1994). Two distinct signals required for sorting and targeting of lysosomal membrane proteins have been identified. First, carboxyl-terminal deletion studies have shown that a dileucine motif in the cytoplasmic domain of mannose-6-phosphate receptors is necessary for efficient sorting of lysosomal hydrolases from *trans*-Golgi to lysosomes, via late endosomes, by mannose-6-phosphate receptors (Johnson and Kornfeld, 1992). Recently, targeting of LIMP II, a resident lysosomal integral membrane protein, to lysosomes has also been shown to be mediated by a Leu-Ile motif in the cytoplasmic tail (Ogata and Fukuda, 1994; Sandoval et al., 1994). Strikingly, there are two consecutive leucine residues within the

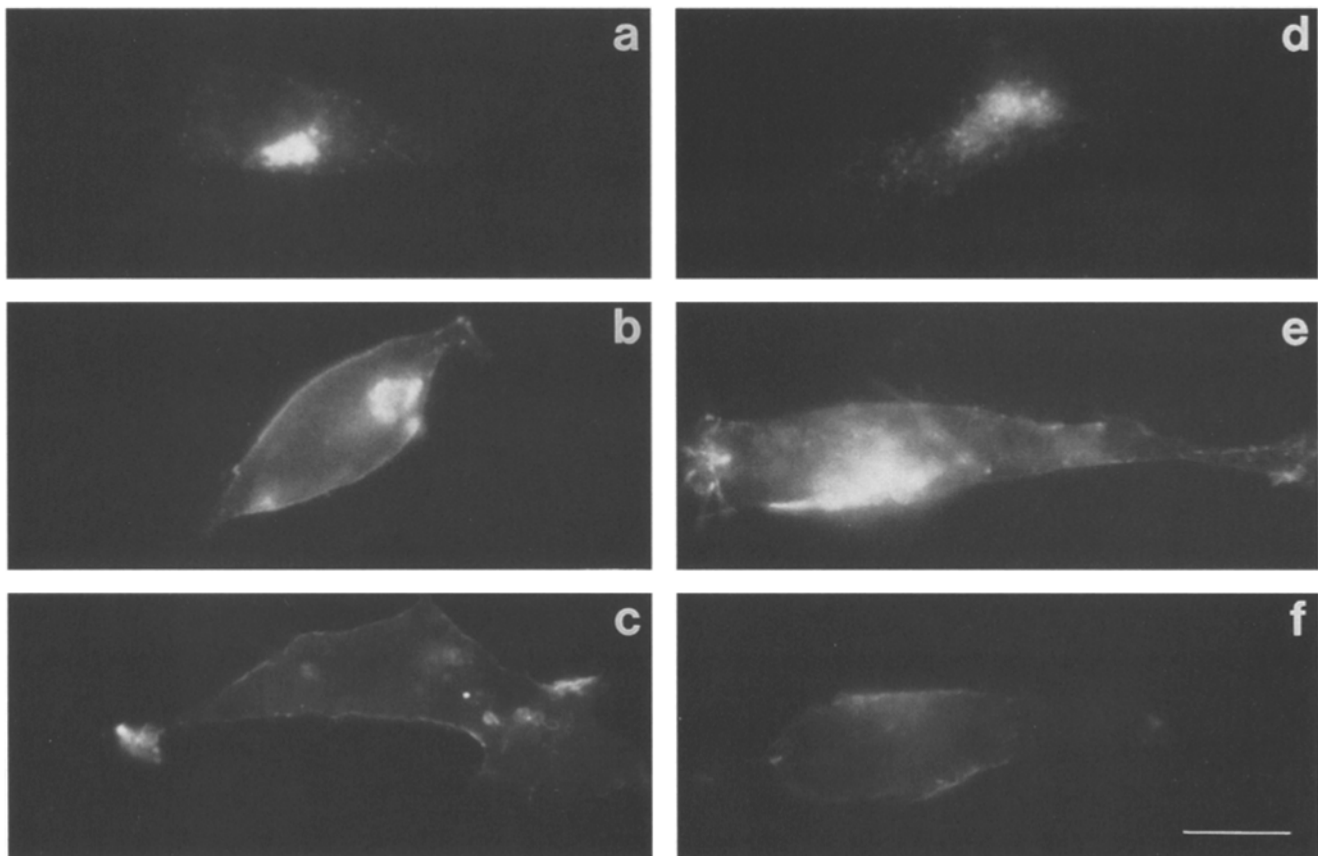


Figure 10. Immunofluorescence microscopy of L cells expressing full-length CD8/gp75 chimeras and carboxyl-terminal deletion mutants of CD8/gp75 chimeras. Cells transfected with CD8/gp75 TM+Cyt (a), CD8/gp75 Cyt (b), CD8/gp75 TM+Cyt ΔC27 (c and e), or CD8/gp75 Cyt ΔC27 (d and f) were cultured for 48 h with (a and b) or without (c-f) leupeptin in the culture medium. Cells were fixed in 2% paraformaldehyde and stained with mAb OKT-8, followed by FITC-conjugated anti-mouse IgG antibody with (a-d) or without (e and f) prior membrane permeabilization. Bar, 16 μm.

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hgp75 (502)  RARRSMD E ANQ P L L T D QYQCYAEEYEK L Q N P N Q S V V
mgp75 (502)  RSRSTKN E ANQ P L L T D H Y Q R Y A E D Y E E L P N P N H S M V
hTyr (502)   H K R K Q L P E E K Q P L L M D K E D Y H S L Y Q S H L
mTyr (505)   K K K K Q P Q E E R Q P L L M D K D D Y H S L L Y Q S H L
Pmel17(624) R-----E - N S P L L S G Q Q V
mP-protein(89) S K D S C F T E - M T P L L R N S L Q E K -----K
hP-protein(85) L K D L S F K E D - T P L L W N S S Q K K -----R
mTRP-2 (495) R L R K G Y A -----P L M E T G L S S K R Y T E E A

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Figure 11. Alignment of carboxyl-terminal amino acid sequences of human (*h*) and mouse (*m*) brown locus protein gp75 (TRP-1), albino locus protein tyrosinase, slaty locus protein dopachrome tautomerase (TRP-2), and the silver locus product Pmel 17. Amino acid sequences in single letter code are shown beginning

at the junction of membrane spanning region (*open boxes*) and the cytoplasmic tail. For the *pink eyed* locus p protein, the sequences shown are part of the first amino-terminal loop predicted to be facing the cytoplasmic side of the melanosomal membrane. Boxes at the end of the p protein sequences are the first transmembrane regions proximal to the amino-terminal end. Amino acid residues conserved in the region of gp75 sorting signal and the glutamic acid (*E*) amino-terminal to the signal are shown in bold within the stippled box. Single dashes and series of dashes represent breaks introduced between consecutive amino acids for the purpose of sequence alignment. Series of dashes interrupted by back slashes represent stretches of amino acids not shown.

gp75 sorting sequence, Asn-Gln-Pro-Leu-Leu-Thr. The sequence motif Pro-Leu-Leu, located 10–12 amino acids from the TM domain in the cytoplasmic tail, is conserved among human and mouse tyrosinases gp75, and the pMel 17 protein, suggesting that the dileucine sequence in the cytoplasmic-tail sequence is critical for sorting melanosomal proteins along the endocytic pathway.

A close examination of the amino-acid sequences of other melanocyte-specific proteins reveals variations of this notion. First, the *p*-locus protein has multiple potential TM domains, and has been proposed to traverse the melanosomal membrane several times (Gardner et al., 1992). The sequence Pro-Leu-Leu is located in the first amino-terminal loop that faces the cytoplasm. This suggests that the position of the tripeptide sequence with respect to the carboxyl end of the protein is not critical. However, the exact membrane orientation of the *p*-locus protein, and the role of the tripeptide sequence in its intracellular sorting need to be investigated. Second, in TRP-2 (the product of the *slaty* locus, and a type I melanogenic protein like tyrosinase and gp75), only Pro-Leu residues of the tripeptide Pro-Leu-Leu, and not the dileucine motif, is conserved, suggesting the existence of alternative or additional sorting signals. In this context, a second signal, a Gly-Tyr motif, has been shown to be critical for sorting of a family of lysosomal glycoproteins (lgps) in the TGN and targeting them to lysosomes (Williams and Fukuda, 1991; Harter and Mellman, 1992; Matthews et al., 1992). In this family of lgps, the Gly-Tyr signal is located in the cytoplasmic tail at least three amino acids from the carboxyl-terminal residue. It is interesting to note that in TRP-2, a Gly-Tyr motif is present at carboxyl-terminal position 19 and 18 (Jackson et al., 1992; Winder et al., 1993). Therefore, sorting and targeting of TRP-2 may be mediated by this Gly-Tyr motif. This raises the possibility that such additional redundant signals could also be present between amino acid 518 and the COOH terminus in gp75 and other melanosomal proteins.

Although the majority of lysosomal membrane proteins are sorted at the Golgi apparatus for intracellular retention and targeting to lysosomes, some lysosomal proteins may be delivered to lysosomes by an alternative route via endocytosis of proteins transported directly to the cell surface (Harter and Mellman, 1992; Matthews et al., 1992). The tyrosine residue in the *trans*-Golgi sorting signal Gly-Tyr in the cytoplasmic tails of lgps also appears to be critical

for internalization of cell-surface lgps, similar to tyrosine signals responsible for receptor-mediated endocytosis. There are multiple tyrosine residues in the cytoplasmic tail of gp75. However, these tyrosine residues do not appear to play a significant role in sorting gp75 along the endocytic pathway by internalization of cell surface gp75, because deletion mutants lacking these tyrosine residues were not detected on the cell surface, but were sorted efficiently to intracellular vesicles.

Some proteins normally present on the cell surface are also known to be targeted to the endosomes/lysosomes under specific physiological or pathological conditions, or when expressed in heterologous cell types. A dileucine motif or a signal containing two adjacent nonaromatic hydrophobic residues is required for the lysosomal targeting of these proteins via endocytic pathway either by *trans*-Golgi sorting along the late endosomal/lysosomal pathway or by internalization from plasma membrane into early endosomes (Letourneur and Klausner, 1992; Pelchen et al., 1992; Aiken et al., 1994; Corvera et al., 1994; Matter et al., 1994; Odorizzi et al., 1994). For example, it has been shown that a dileucine motif in the cytoplasmic tail of CD3 γ and CD3 δ chains of the T cell surface antigen receptor complex is responsible for the *trans*-Golgi sorting to lysosomes. The same motif is required for the endocytosis and lysosomal degradation of the resident T cell surface protein CD4, induced by human immunodeficiency virus 1 protein nef in T cells. However, newly synthesized CD3 T cell receptor chains, and CD4 are transported to the T cell surface despite the presence of a dileucine motif. This suggests the existence of mechanisms or additional signals that either suppress the dileucine signal during biosynthetic transport or activate the signal for internalization (Letourneur and Klausner, 1992; Pelchen-Matthews et al., 1992). Therefore, it is the context, and not mere presence, of the dileucine motif that appears to determine targeting of proteins to the endosome/lysosome compartment. The fate of proteins entering this pathway then depends, presumably, on sorting events within the endosomal compartment. Although gp75 and lysosomal proteins share sorting signals, in melanocytic cells, additional signals may operate either to target gp75 to distinct melanosomal precursors or to cause retention in endosomes (“modified endosomes”) for biogenesis of melanosomes. In this context, it is of interest to note that in addition to the dileucine motif, a glutamic acid located two or three amino acids amino-

terminal to the Pro-Leu-Leu motif is also conserved in all melanocyte-specific proteins, except TRP-2. In the absence of the dileucine signal critical for sorting along endosomal/lysosomal pathway, truncated gp75 containing only the glutamic acid signal is transported to the cell surface. Alternatively, it is possible that additional residues amino-terminal to the Pro-Leu-Leu motif are also critical for intracellular sorting of gp75. These membrane-proximal amino acid residues may play an indirect role by extending the sorting signal into the cytosol for interaction with the protein components of sorting and targeting machinery.

Sorting of gp75 and the CD8/gp75 chimeras along the endosomal/lysosomal pathway has implications for targeting of melanosomal proteins in melanocytic cells and the biogenesis of melanosomes. Sorting of melanosomal proteins along the endosomal/lysosomal pathway could indicate that lysosomes and melanosomes share a common precursor, and that in melanocytic cells melanosomes ("specialized endosomes") evolve from this common precursor. Or, it could indicate biogenesis of two distinct organelles by sorting of proteins along a shared pathway that then diverges to deliver proteins to distinct organelle precursors. Melanosomes and lysosomes have several biochemical characteristics in common with late endosomes. The pH of the lumen of both organelles is acidic (Moellmann et al., 1989; Bhatnagar et al., 1993). The enzyme acid phosphatase, a lysosomal marker, and other hydrolases have been reported to be also present in melanosomes (Seiji and Kikuchi, 1969; Boissy et al., 1987). In mouse melanoma cells, gp75 and LAMP-1 have been shown to colocalize to melanosomal precursors that morphologically resemble endosomes (Zhou et al., 1993). There are potential distinctions in intracellular routes of targeting proteins to these two organelles. The targeting of melanosomal proteins, and the movement of melanosomal membrane proteins appears to be predominantly unidirectional, i.e., after biogenesis of melanosomes, melanin pigment accumulates in the lumen, and eventually the whole organelle is exported into neighboring keratinocytes. Lysosomes receive input from multiple pathways, i.e., in addition to proteins delivered directly from the trans-Golgi via endosomes, proteins are also transported from the cell surface to lysosomes for degradation or retention as resident membrane proteins. Lysosomal membrane proteins may also recycle between endosomes and cell surface (Trowbridge et al., 1993).

Identification of the dileucine-containing sequence as a sorting signal in melanosomal proteins, together with the shared features of late endosomes/lysosomes and melanosomes, argues in favor of the notion that melanosomes are specialized endosomes (Zhou et al., 1993). Our data show that the dileucine-containing sequence is critical for sorting and intracellular retention of gp75, indicating that sorting signals and pathways of biogenesis may be shared between melanosomes and lysosomes.

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