A role for glycosphingolipids in protein sorting

Sprong, H.

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Chapter 5

Glycosphingolipids are required for sorting of melanosomal proteins in the Golgi complex

Hein Sprong, Sophie Degroote, Tijs Claessens, Judith van Drunen, Viola Oorschot, Ben H.C. Westerink\(^a\), Yoshio Hirabayashi\(^b\), Judith Klumperman, Peter van der Sluijs, and Gerrit van Meer

\(^a\)Department of Medicinal Chemistry, Center for Pharmacy, University of Groningen, Groningen, The Netherlands
\(^b\)Laboratory for Memory and Learning, RIKEN Brain Science Institute, Saitama, Japan

Summary
Glycosphingolipids are ubiquitously expressed and essential for embryonic development. Mouse mutant GM95 cells survive without glycolipids. Parental melanoma cells are black, but GM95 cells are white. In these cells the first enzyme in melanin synthesis, tyrosinase, was not in melanosomes but accumulated in the Golgi. Tyrosinase with a lengthened transmembrane domain and tyrosinase-related protein 1 (TRP-1) reached melanosomal structures via the plasma membrane, not via the intracellular route from the Golgi. Biosynthetic transport of lysosomal enzymes was unaffected. Intracellular tyrosinase and TRP-1 transport was restored upon transfection of ceramide glucosyltransferase. Glucosylceramide, synthesized on the cytosolic surface of the Golgi where melanosomal proteins are sorted by an adaptor-mediated mechanism, may be essential for the budding of vesicles destined for specific secretory compartments.
Introduction

Glycosphingolipids consist of a carbohydrate moiety that is attached to ceramide, a lipid anchor with two hydrophobic tails. Glycosphingolipids principally differ from the more abundant glycerophospholipids that have two fatty acyl chains esterified to a glycerol backbone, since the single fatty acid in ceramide is anchored directly via an amide linkage to the C2 of a long-chain sphingoid base. In addition, ceramides contain free hydroxyl groups close to the carbohydrate moiety, and their fatty acid tends to be longer and more saturated than the fatty acid at the glycerol C2 position of the glycerophospholipids. Due to these structural differences, the affinity between glycosphingolipids is usually higher than between glycerophospholipids.

The most simple glycosphingolipids are glucosylceramide (GlcCer) and galactosylceramide (GalCer). GlcCer occurs in all mammalian cells. It serves as the basis for a large number of complex glycosphingolipids. GalCer, in contrast, is expressed in specialized cells and can be sulfated or galactosylated, but is generally not further modified. Several cellular membranes contain high glycosphingolipid levels. In myelin and the apical membrane of some epithelial cells glycosphingolipid may constitute up to 20-35 mol% of total lipid. The high glycosphingolipid concentration in these plasma membranes is thought to serve insulating and protective functions.

The diverse chemical structure of complex glycosphingolipids suggests that they are involved in cell-cell and cell-substratum interactions (194). Although such interactions have been the subject of many studies, surprisingly little is known about their function in individual cells. Importantly, sphingolipids, and in particular glycosphingolipids, have the propensity to cluster in an environment of other lipids (259, 260). Some of their functions may therefore be explained by the ability to form lateral microdomains with physicochemical properties that are distinct from those of the bulk membrane (194, 261). In 1988, we proposed that lateral domains of glycosphingolipids in the trans-Golgi network (TGN) are involved in the sorting of membrane proteins (103). The ubiquitous expression of glycosphingolipids suggests that they exert organizing functions in all eukaryotic cells (262).

Knock-out mice with null alleles for ceramide glucosyltransferase (CGlcT) lack GlcCer-based glycolipids and die at embryonic day 7.5 (224). The fact that individual embryonic cells are viable and undergo a minimal differentiation program, confirms the notion that glycosphingolipids are essential for multicellular organisms. In addition, no apparent phenotype has been reported for the GM95 melanoma cell line which lacks CGlcT activity (263). Thus, neither in vivo nor in vitro models for glycolipid deficiency, so far, suggested a function for these lipids in the individual cell. In contrast, we here describe a dramatic phenotype for the GM95 cells: GM95 cells are unable to synthesize melanin pigment. Our data show that the glycolipid-deficient GM95 cells are defective in intracellular transport of melanosomal proteins from the Golgi complex to melanosomes.

Results

Sphingolipid composition and pigmentation of the cell lines

When passaging the glycolipid-negative GM95 cells and the parental MEB4 cells, we noticed a striking difference. While the MEB4 cell pellet was black, the pellet of GM95 cells was white. The degree of pigmentation of the MEB4 cells increased 4-fold when 1 mM L-tyrosine was added to the growth medium (264), confirming that the black color of MEB4 melanoma cells is due to the pigment melanin of which L-tyrosine is the precursor. The GM95 cells remained white in the presence of L-tyrosine, and thus did not synthesize melanin (Figure 1).
Figure 1: Sphingolipid composition and pigmentation of melanoma cells

A: Cells were labeled with [³H]sphingosine for 48 h. Lipids were extracted, separated by acidic thin layer chromatography, and visualized by fluorography. Spots were scraped and quantified by liquid scintillation counting. Incorporation of [³H] (x10³ dpm) in MEB4 cells, GM95 cells transfected with empty vector (mock), with cDNA encoding CGlcT (CGlcT), and with CGlcT containing an ER-retrieval signal (CGlcT-KKV), respectively: GlcCer: 51, not detectable (ND), 0.7, and 25; GM3: 22, ND, 1.2, and 6; sphingomyelin (SM): 47, 84, 96, and 54. GM95 cells transfected with GalT-1: GalCer: 6.5; SM: 97, n=4; background: 0.2; ND: < 0.4. B: Cells were scraped, pelleted in a microtiter plate and photographed. Next, cell pellets were solubilized and pigment was measured colorimetrically (A₄₅₃/mg protein; data from a representative experiment in quadruplicate; SD= 0.01; CHO cells: 0.03).
GM95 cells lack CGlC activity and lipid analysis confirmed (Figure 1A) that MEB4 cells produced GlcCer, lactosylceramide and sialyllactosylceramide (GM3), while GM95 cells did not synthesize glycosphingolipids (263). Partial restoration of glycolipid synthesis was achieved by stable transfection of GM95 cells with CGlC cDNA, which also returned pigmentation as shown in Figure 1B. Both glycolipid synthesis and pigmentation increased dramatically in GM95 cells transfected with a cDNA encoding CGlC with the ER-retrieval signal KKVK (Figure 1A, B). In this cell line, CGlC was relocated from the Golgi to the endoplasmic reticulum (ER), as shown by subcellular fractionation (Figure 1C; cf. (122). Also transfection with ceramide galactosyltransferase (GalT-1) cDNA restored pigmentation (Figure 1B; see below). Thus, pigmentation directly correlated with synthesis of glycosphingolipids.

Table 1: Oxidation of tyrosine to L-DOPA by cultured cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>L-DOPA (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>ND</td>
</tr>
<tr>
<td>MEB4</td>
<td>76 ± 32</td>
</tr>
<tr>
<td>GM95-mock</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>GM95-CGlC-KKVK</td>
<td>70 ± 33</td>
</tr>
<tr>
<td>GM95-tyrosinase-TM6</td>
<td>79 ± 38</td>
</tr>
</tbody>
</table>

L-DOPA was measured in cells (3 d) and medium as described under Materials and methods. Since L-DOPA is unstable at pH 7.4, the assay likely yields underestimates of cellular L-DOPA concentrations. GM95 cells were transfected with empty vector (mock), CGlC with ER-retrieval signal (CGlC-KKVK), and tyrosinase with lengthened transmembrane domain (tyrosinase-TM6; Figure 5). Considerable amounts of L-DOPA were found in medium of MEB4, GM95-CGlC-KKVK, and GM95-tyrosinase-TM6 cells (1.2 ± 0.4 nmol/10^6 cells), whereas L-DOPA was not detectable in medium of CHO or GM95 cells (< 0.01 nmol/10^6 cells). ND: not detectable: < 0.5 pmol/10^6 cells. Values are from 2 independent experiments.

**GM95 cells contain active tyrosinase, but do not make L-DOPA**

To identify the molecular basis of the pigmentation defect in GM95 cells, we next measured the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase in vivo, the first and rate-limiting step in melanin synthesis in the melanosome. In contrast to MEB4 cells, GM95 cells produced very little L-DOPA (Table 1). Subsequent oxidation of L-DOPA and polymerization into melanin were retained in GM95 cells, as the cells turned black after incubation with exogenous L-DOPA (Figure 2A). The pigmentation defect in GM95 cells therefore appeared to be at the level of tyrosinase. Indeed, treatment of MEB4 cells with N-
butyldexyonojirimycin, a potent inhibitor of the ER α-glucosidases and of maturation of tyrosinase into the active conformation (265), completely inhibited melanin formation unless exogenous L-DOPA was added to bypass the requirement for tyrosinase (Figure 2B). These results suggested that tyrosinase was not expressed, or not active in GM95 cells. Western blot analysis showed that GM95 cells expressed the same amount of tyrosinase (70 kDa) as MEB4 cells (Figure 2C). In addition, tyrosinase was as active in GM95 cells as in MEB4 cells, as shown by its L-DOPA oxidase activity on gel (Figure 2C). Importantly, this figure also showed TRP-1 dependent L-DOPA oxidase activity (75-80 kDa). Despite its in vitro activity, tyrosinase was unable to make L-DOPA in glycosphingolipid-deficient cells.

**Figure 2: Pigmentation machinery of MEB4 and GM95 cells**

A: Cells were incubated with or without 1 mM L-DOPA, the product of tyrosinase, for 3 h at 37°C, scraped and pelleted into a microtiter plate. B: To inactivate tyrosinase MEB4 cells were pretreated for 3 d with 0.5 mM N-butyldexyonojirimycin (NB-DNJ). C: Equal amounts of protein were resolved by SDS-PAGE and detected by Western blotting using anti-pep7. To measure in gel DOPA oxidase activity, non-boiled samples were run under non-reducing conditions on a 10% gel and analyzed as described. The DOPA oxidase activity in the higher molecular weight band corresponds to TRP-1.

Tyrosinase is not localized in melanosomes in GM95 cells

Comparison of similar regions in MEB4 and GM95 cells by electron microscopy on plastic sections (Figure 3A, C) showed that although GM95 cells contain many endosome- and melanosome-like vacuoles, the characteristic dark melanin pigmentation was absent. When we investigated the localization of the downstream reactions in melanin biosynthesis by preincubating cells with L-DOPA before fixation, MEB4 cells displayed an increase in the number of pigmented melanosomes (Figure 3B). In GM95 cells the L-DOPA treatment resulted in pigment deposition in the vacuolar compartments (Figure 3D). Notably, pigmentation never occurred in the Golgi complex. These morphological experiments
suggested that melanosomal proteins involved in later steps of pigmentation were still transported to post-Golgi vacuoles in GM95 cells.

**Figure 3: Electron microscopy of Epon sections**

MEB4 and GM95 cells were incubated for 3 h at 37°C in the absence (A, C) or presence of 1 mM L-DOPA (B, D) prior to fixation. Comparable regions of the cell were selected for illustration. **A:** In MEB4 cells, melanosomes are readily recognizable by their dark melanin content. The arrowheads point to endosome-like compartments with the same size and shape as melanosomes, but which lack melanin pigment. **B:** Incubation with L-DOPA increased the number of melanin containing compartments. **C:** GM95 cells contain many endosome-like compartments, but lack melanin. **D:** Incubation of GM95 cells with L-DOPA induces the appearance of pigmented organelles, suggesting that the compartments involved in melanin formation are present in these cells, but that the production of melanin is impaired. N = nucleus, M = mitochondrion. Bar: 500 nm.
Figure 4: Localization of tyrosinase

MEB4 (A-C), GM95-mock (D-F, G-I), and GM95-CGlT-KKVK (J-L) cells were fixed, and labeled with rabbit anti-tyrosinase antiserum anti-pep7 (A, D, G, J) and, to mark the Golgi complex, with mouse anti CTR433 antibody (B, E, K) or anti myc-sialyltransferase (H). Cells were counterstained with FITC-labeled goat anti-rabbit (A, D, G, J) and Texas red-labeled goat anti-mouse (B, E, H, K) antisera. Coverslips were analyzed by confocal fluorescence microscopy. Areas of overlapping distributions in the same optical section appear as yellow in the merged images (C, F, I, L). Bar is 10 μm.

Tyrosinase is inactive outside melanosomes (266). To determine whether tyrosinase was not localized to melanosomes in GM95 cells, we investigated its distribution by double-label immunofluorescence microscopy using antibodies against tyrosinase, the medial-Golgi marker CTR433 (210), and the myc-tagged trans-Golgi marker sialyltransferase (267). Tyrosinase was predominantly localized to punctate cytoplasmic structures in MEB4 cells. In addition, we found some tyrosinase in the perinuclear region as shown in Figure 4A-C. This labeling pattern is typical for melanosomes as illustrated by electron micrographs of MEB4 cells (Figure 3). In striking contrast, antibodies against tyrosinase hardly labeled peripheral structures in GM95 cells, and labeling was essentially limited to the perinuclear region (Figure 4D, G). The distribution of tyrosinase in the GM95 cells was closely similar, but not identical to that of the medial- and trans-Golgi markers (Figure 4D-F, 4G-I). Since pigmentation was restored in the GM95 transfectant expressing CGlT-KKVK, we also analyzed the localization of tyrosinase in this cell line. Consistent with the ability of this transfectant to synthesize L-DOPA and pigment, we found a large fraction of tyrosinase localized to peripheral structures outside the Golgi area (Figure 4J-L) as in the MEB4 cells. The localization of tyrosinase in the Golgi area of GM95
cells suggested that it was not associated with other elements of the pigmentation machinery in functional melanosomes, explaining the lack of pigment in GM95 cells.

**Lengthening the transmembrane domain of tyrosinase restores pigmentation**

The localization of tyrosinase in the Golgi complex of GM95 cells suggests that the sorting information in the protein needed for transport out of the Golgi complex is no longer recognized and that a secondary signal is responsible for Golgi arrest. One typical Golgi retention signal is a short transmembrane domain of ~17 amino acids (268), and indeed mouse tyrosinase has a predicted transmembrane domain of 17 amino acids (269). To investigate whether this domain was responsible for its retention in the Golgi complex, we generated stable GM95 transfectants expressing mouse tyrosinase in which the transmembrane domain was lengthened with the 6 hydrophobic amino acids VLALVA, between A490 and A491 (tyrosinase-TM6). Even GM95 cells with a low (2-fold) overexpression of tyrosinase-TM6 produced L-DOPA very efficiently (Table 1), and regained the ability to produce pigment as documented by the black cell pellet in Figure 5A. Consistent with this observation, tyrosinase-TM6 localized to vacuolar structures in the cytoplasm (Figure 5B). To rule out the possibility that overexpression caused saturation of the sorting machinery in the Golgi complex and allowed tyrosinase to escape, we generated a stable GM95 transfectant with a similar expression level of wild-type tyrosinase. These cells remained white and the transfected tyrosinase was localized to the Golgi complex (Figure 5A, B) like endogenous tyrosinase (Figure 4D). Only 4-6 fold overexpression of tyrosinase caused a minimal amount of pigmentation and tyrosinase distribution outside the Golgi area. The results in the tyrosinase-TM6 and tyrosinase GM95 transfectants showed that the pigmentation defect in cells without glycolipids is solely due to mislocalization of tyrosinase, and that its arrest in the Golgi complex is due to a cryptic Golgi retention signal.

Tyrosinase is thought to be transported directly from the Golgi complex to melanosomes. A lengthened transmembrane domain could have restored transport to peripheral vacuoles by targeting tyrosinase-TM6 into this direct pathway. Alternatively, tyrosinase-TM6 might be transported to the plasma membrane and internalized by endocytosis. To discriminate between these possibilities, MEB4 and GM95 cells were stably transfected with myc-tagged tyrosinase (tyrosinase-myc) and tyrosinase-TM6 (tyrosinase-TM6-myc). The appearance of newly synthesized proteins on the cell surface was determined in pulse chase experiments by cell surface biotinylation. We used myc-tagged constructs for these experiments since anti-pep7 antibody did not efficiently immunoprecipitate tyrosinase. The epitope tag did not affect the steady state distributions of tyrosinase and tyrosinase-TM6 (Figure 5A, B). However, a striking difference was observed in the appearance of newly synthesized tyrosinase-myc and tyrosinase-TM6-myc on the surface of the two cell lines. Little tyrosinase-myc was found on the surface of GM95 cells (Figure 5C), where the protein localized in the Golgi complex (Figure 5B). Similarly, little of the tyrosinase-myc was observed on the surface of MEB4 cells at any chase-time suggesting transport from the Golgi complex to the melanosome via a direct, intracellular pathway. In contrast, the amount on the surface significantly increased with time for tyrosinase-TM6-myc in GM95 cells (Figure 5C). This suggested that lengthening the transmembrane domain of tyrosinase resulted in incorporation of tyrosinase-TM6 into a vesicular pathway from the Golgi complex to the plasma membrane in GM95 cells. No significant fraction on the surface was observed for tyrosinase-TM6-myc in MEB4 cells. This suggested that the signal responsible for tyrosinase-TM6-myc transport in the direct pathway is dominant over that of the plasma membrane route. In GM95 cells, the signal for transport to the melanosome is either non-functional or the pathway no longer exists.
Figure 5: Localization and transport of tyrosinase ± TM6

A: Pellets in microtiter plates of GM95 cells transfected with empty vector (Mock), tyrosinase (Tyr), tyrosinase with lengthened transmembrane domain (Tyr-TM6), and their myc-tagged versions (Tyr-myc and Tyr-TM6-myc). Both GM95-Tyr and GM95-Tyr-TM6 cells expressed tyrosinase at levels 2-3 times the level in GM95 cells by Western blotting.

B: The distribution of tyrosinase was analyzed by confocal fluorescence microscopy using the anti-pep7 antibody or the anti-myc antibody and a FITC-labeled secondary antibody. Anti-pep7 labels both endogenous tyrosinase in the Golgi of these cells (conform figure 4), and transfected tyrosinase. C: Cells transfected with tyrosinase-myc or tyrosinase-TM6-myc were pulse-labeled for 60 min with Tran[^35]S]label, chased for the indicated time (min), and biotinylated on ice. Tyrosinase was immunoprecipitated from detergent lysates with the anti-myc antibody. Immunoprecipitated protein was eluted from the beads, and part was analyzed by SDS-PAGE and autoradiography (Cells). Biotinylated tyrosinase was immunoprecipitated from the remainder using streptavidin-agarose beads and analyzed by SDS-PAGE and phosphor imaging (Surface). Tyrosinase (~70 kDa) at the surface was quantified by subtracting a blank value (b) from the signal (s) in each lane, and was divided by the signal of the cells at t=0 in a
phosphor image obtained under the same conditions (not shown), and expressed as percent of total at t=0. Tyrosinase is rapidly degraded. This occurs in the ER and is due to inefficient folding (304). Data are the mean of 2 independent experiments, error bars: range.
TRP-1 reaches peripheral vacuoles in GM95 cells via the cell surface

Addition of L-DOPA to GM95 cells resulted in pigment synthesis in peripheral vacuoles (Figure 2, 3), implying that a DOPA-oxidase activity other than tyrosinase must be present in these organelles. Because this activity in melanocytes is typically due to the melanosomal protein TRP-1 (Figure 2C), we investigated the distribution of endogenous TRP-1 in GM95 cells. TRP-1 was concentrated in punctate structures throughout the cytoplasm (Figure 6A). Most likely, TRP-1 is transported from the Golgi complex to melanosomes via the direct pathway (270, 271). To investigate whether TRP-1 still followed the direct pathway in GM95 cells, its transport route was established in a pulse-chase and cell surface biotinylation experiment (Figure 6B). The rate of synthesis and glycosylation of TRP-1 were indistinguishable between MEB4 and GM95 cells, indicating that biosynthetic transport through the ER and Golgi complex was not affected in GM95 cells. Also the rate of degradation, approximately 20% in 60 min, was identical in the two cell lines. In contrast, the fraction of newly synthesized TRP-1 present on the cell surface at various time-points during 1 h of chase increased 6-fold in GM95 cells as compared to MEB4 cells. The fraction of TRP-1 on the plasma membrane of the GM95 transfectant expressing CGlcT-KKVK was reduced to similar levels as in MEB4 cells (Figure 6B).

The increased presence of TRP-1 on the surface of GM95 cells suggested that TRP-1 is not transported directly from the Golgi complex to melanosomes, but reached the melanosome via endocytosis from the plasma membrane. We tested this hypothesis in an independent experiment in which MEB4 and GM95 cells were incubated at 37°C with TA99, an antibody against the exoplasmic portion of TRP-1. The relative amount of endocytosed antibody molecules was then determined by Western blot of cell lysates. In three experiments, 4-6 times more TA99 was taken up by the GM95 cells than by MEB4 cells. In endocytosis experiments with an irrelevant antibody no cell-associated antibody was detected in either GM95 or MEB4 cells (Figure 6C). These data show enhanced transport of TRP-1 via the cell surface to melanosomes in GM95 cells compared to MEB4 cells. Immunofluorescence microscopy on parallel dishes with FITC-labeled goat anti-mouse antibody confirmed that the bulk of internalized antibody resided in peripheral endocytic compartments (not shown). The predicted transmembrane domain of TRP-1 comprises 24 amino acids (269). As in the case of tyrosinase-TM6-myc, the long transmembrane domain appears to function as a plasma membrane signal only in the absence of glycosphingolipids.

Transport of lysosomal enzymes to lysosomes is unchanged in GM95 cells

Melanosomes are considered to be specialized endosomes/lysosomes. Two direct pathways from the Golgi complex to endosomes are known. They select their cargo through interactions between an adaptor-protein complex (AP-1 and AP-3) and a sorting signal in the cytoplasmic tails of cargo proteins. Both tyrosinase and TRP-1 contain an AP-3 signal and are missorted in GM95 cells, indicating a defect in AP-3 mediated sorting. The best documented examples of cargo molecules transported via the AP-1 dependent pathway are the two mannose 6-phosphate receptors (MPRs; see 105, transmembrane proteins that mediate transport of most soluble lysosomal enzymes to endosomes; 272). The small fraction of lysosomal enzymes that fails to bind to the MPRs, is secreted, and partially recaptured after binding to MPRs on the cell surface. Fibroblasts deficient in the μ1A subunit of AP-1 missort cathepsin D resulting in a three-fold increase in the release of cathepsin D precursor forms into the medium (273). Thus, a defect in the AP-1 pathway is predicted to cause missorting of lysosomal enzymes transported via the MPRs.
Figure 6: Localization and transport of TRP-1

A: Cells were fixed, permeabilized and incubated with the anti-pep1 antibody against TRP-1, counterstained with FITC-labeled secondary antibody, and viewed by confocal immuno-fluorescence microscopy. Bar: 10 μm. B: Cells were pulse-labeled for 15 min, chased for the indicated time (min), and biotinylated like tyrosinase in figure 5, but using the TA99 antibody to immuno-precipitate TRP-1. Data (phosphor images) are representative of 3 experiments. IM: immature, core-glycosylated form, M: mature, complex-glycosylated forms. C: Cells were incubated with TA99 antibody against the exoplasmic domain of TRP-1 or with the control mouse anti-myc monoclonal 9E10 for 3 h at 37°C. After washing, internalized antibody was visualized by SDS-PAGE and Western blotting using anti-IgG coupled to horseradish peroxidase. Reactivity of 9E10 with the anti-IgG was controlled by a Western blot of myc-tagged sialyltransferase (not shown). To allow a quantitative comparison, different amounts of each lysate were loaded on the gel, and as an internal control the total amount of TRP-1 in the samples (present as mature and immature forms) was measured by Western blotting using anti-pep1.
We next investigated whether the AP-1 pathway was affected in GM95 cells by assaying secretion of two lysosomal hydrolases that are known to bind to MPRs (274). About 20% of β-hexosaminidase and β-galactosidase was secreted in 36 h (Figure 7A). In the presence of mannose 6-phosphate, secretion increased 2-fold in both MEB4 and GM95 cells. In addition, we determined transport (and maturation) of newly synthesized cathepsin D to lysosomes, and its secretion into the medium. In the media of both cell lines, two immature forms of cathepsin D (51 and 53 kDa) were detected, probably precursors (p) with different N-glycans (Figure 7B). Both cell lysates contained a small amount of the precursors. The main form present was the 44 kDa intermediate (i), which results from a first cleavage in endosomes, whereas very little mature cathepsin D (m; 31 kDa) was detected. The amount of cathepsin D secreted as percent of the total forms was the same in both cell lines (18 ± 1% for MEB4, 20 ± 1% for GM95). In the presence of 5 mM mannose 6-phosphate, a two-fold increase in the secretion of the 51 and 53 kDa precursors was observed (35 ± 4% for MEB4, 37 ± 6% for GM95), showing that in both cell lines half of the cathepsin D which is secreted is recaptured by MPR-mediated endocytosis. The unchanged secretion of three hydrolases and the identical effect of mannose 6-phosphate in GM95 and MEB4 cells showed that the AP-1 pathway in GM95 cells was unaffected by the absence of glycolipids.

Stimulation of glycosphingolipid synthesis increases pigmentation
CGlcT transfers glucose from UDP-glucose to ceramide and thus produces glycosphingolipids at the expense of ceramide. Pigmentation might therefore require the production of glycosphingolipids or, alternatively, the removal of ceramide. Indeed, ceramide has been reported to inhibit glycoprotein traffic through the Golgi (275). If the block in transport of tyrosinase in GM95 cells would be caused by increased ceramide levels, addition of exogenous ceramide should inhibit tyrosinase transport and pigmentation even more. However, the opposite was observed. When MEB4 or GM95-CGlcT cells were incubated with exogenous ceramide, pigmentation increased (Figure 8B). Ceramide addition furthermore increased the synthesis of glycosphingolipids and sphingomyelin (SM) as shown in Figure 8A. In GM95 cells, ceramide addition enhanced SM synthesis but not pigmentation. Thus, pigmentation did not correlate with ceramide or SM concentrations but depended on the level of glycosphingolipids.

We next investigated whether galacto-glycosphingolipids could substitute for the gluco-glycosphingolipids in pigment formation. GalCer is structurally related to GlcCer and is synthesized exclusively in the ER by GalT-1 (chapter 3). Neither MEB4 nor GM95 cells contain GalCer (Figure 1). In GM95 cells stably transfected with GalT-1 cDNA, we found significant amounts of GalCer (Figure 1A) and galactosyldiglycerides (not shown) but no higher glycosphingolipids. GM95-GalT-1 cells produced melanin pigment (Figure 1B). GalT-1 is not related to CGlcT. This suggested that the enzymatic activity of the two unrelated proteins is required to restore pigment formation, and that GalCer can substitute for GlcCer to fulfil the glycolipid requirement in targeting melanosomal proteins from the Golgi complex to melanosomes. Finally, exogenous ceramide increased both GalCer synthesis and pigmentation in the GM95-GalT-1 cells (Figure 8), which corroborates the notion that glycolipid synthesis and not ceramide removal is required for tyrosinase transport out of the Golgi complex.
Figure 7: Secretion of lysosomal enzymes
A: Cells were cultured for 36 h in the presence or absence of 5 mM mannose 6-phosphate (Man6P), and the activities of β-hexosaminidase and β-galactosidase were determined in the media and in the cells. Tissue culture medium and lysis buffer were used as background. Signals were 30-40 times over background. Data are the mean of 2 experiments (n=4). B: Cells were pulse-labeled with \([^{15}S]\)labeled amino acids for 60 min, and chased in the presence or absence of 5 mM mannose 6-phosphate for 3.5 h. Cathepsin D was immunoprecipitated from the media and detergent lysates with the anti-cathepsin D antibody. Immunoprecipitated proteins were analyzed by SDS-PAGE and phosphor imaging. The precursors (p), intermediate (i), and mature (m) forms of cathepsin D are indicated. Numbers indicate the mean percentage of cathepsin D secreted into the medium of 3 independent experiments (SD < 6%).
Figure 8: Ceramide stimulates glycosphingolipid synthesis and pigmentation
A: Cells were incubated with D-[1-14C]galactose in the absence or presence of 20 μM bovine brain ceramides for 3 d and incorporation of [14C]galactose into glycosphingolipids was expressed as percentage of total [14C]lipids. The sphingomyelin content of all cells incubated with ceramides increased 1.3 ± 0.1 fold (not shown). B: Pigmentation was measured and expressed as in figure 1. Data are means of triplicate experiments.

Discussion
Mislocalization of tyrosinase is responsible for the absence of pigment from GM95 cells
Here we report that the loss of pigmentation in the melanoma mutant cell line GM95 is specifically due to a block in the first step in melanin synthesis, the conversion of tyrosine to L-DOPA by tyrosinase. Tyrosinase is not required for the subsequent reactions, since addition of L-DOPA to melanoma cells in which tyrosinase had been inactivated by N-butyldeoxynojirimycin, where TRP-1 is still active, allowed these cells to form pigment (276). The
same was observed in GM95 cells treated with the glucosidase inhibitor N-butyldIDEOXYNOJIRIMICIN (Figure 2B). In addition, GM95 cells converted exogenous L-DOPA to melanin in structures (Figure 3) that were essentially devoid of tyrosinase (Figure 4). In vivo, tyrosinase activity may be affected in several ways. First, its expression level may vary due to changes in synthesis (264, 277) or turnover (278). Second, tyrosinase may be inactive due to a mutation of the active site (279). Finally, various defects in transport of tyrosinase to the melanosomes affect pigmentation. In the platinum mouse, a mutation truncates the cytoplasmic tail of tyrosinase. The truncated tyrosinase bypasses the melanosomes, which results in severe ocucutaneous albinism (266). In contrast, in amelanotic human melanoma cells wild-type tyrosinase is more efficiently retained in the ER and degraded by the proteasome (280). In the present study, a defect in glycosphingolipid synthesis caused retention of tyrosinase in the Golgi complex and possibly the TGN (Figure 4), and abrogated pigmentation (Figure 1), whereas this tyrosinase was fully active in vitro. From these observations we conclude that the lumenal environment of the Golgi complex is not suited for tyrosinase to perform its biochemical function. One obvious difference between the Golgi complex and melanosomes is the exclusive presence in the melanosomes of a transporter that allows tyrosine to enter the lumen where the active center of tyrosinase is located. Selective relocalization of tyrosinase to the melanosomes by a mutation in its transmembrane domain restored pigmentation (Figure 5). This shows that tyrosinase mislocalization itself was responsible for the pigmentation loss in GM95 cells.

Melanosomal protein sorting in the absence of glycosphingolipids

A di-leucine containing motif in the cytosolic tail is required for proper targeting of a number of melanosomal membrane proteins including tyrosinase and TRP-1 (266, 270, 281, 282). The tyrosinase di-leucine motif specifically interacts with the AP-3 adaptor complex (283), and not with AP-1, a distinct adaptor at the TGN involved in sorting membrane proteins towards endosomes (284). The significance of AP-3 in sorting tyrosinase to the melanosome is probably best illustrated by the pearl mouse, where a mutation in the β3A subunit of AP-3 causes hypopigmentation (285). Downregulation of AP-3 levels with antisense oligonucleotides (105) or in Hermansky-Pudlack Syndrome patients lacking the β3A subunit (286) redirects AP-3 dependent lysosomal membrane proteins to the cell surface. We found a comparable effect for TRP-1 and tyrosinase-TM6 in glycosphingolipid deficient cells. Apparently, in the absence of glycosphingolipids protein sorting in the AP-3 pathway is disrupted, or the AP-3 pathway no longer operates. In contrast, glycosphingolipids were not required for the AP-1 pathway from the Golgi to the endosomes.

The compartments reached by TRP-1 and tyrosinase-TM6 in the GM95 cells (Figure 5, 6) are indistinguishable from melanosomes in MEB4 cells at the light microscopical level, and probably the same organelles turned electron-dense upon addition of exogenous L-DOPA (Figure 3). However, our preliminary evidence from ultrastructural immuno-localization experiments with several marker proteins suggests that melanosomes can be discriminated from lysosomes in MEB4 cells but are no longer separate compartments in GM95 cells. This suggests that the proper organization of the secretory/endocytic system depends on an active AP-3 pathway.

By what mechanism do glycosphingolipids enable tyrosinase transport?

The primary defect in GM95 cells is the lack of glycosphingolipid synthesis due to the absence of the CGlcT. Transfection of the cells with either CGlcT or GaIT-1 restored transport and sorting of tyrosinase and TRP-1, DOPA synthesis and pigmentation (Figure 1, 4, 6, and Table 1). In addition, exogenous ceramide stimulated pigmentation (Figure 8). This has led us to
conclude that CGlcT and GalT-1 restored tyrosinase transport by producing glycosphingolipids rather than by removing inhibitory ceramide.

One function for glycosphingolipids in membrane protein sorting has been proposed for the transport pathway towards the apical plasma membrane domain of epithelial cells. In this model (103), glycosphingolipids spontaneously aggregate with cholesterol into lateral domains or 'rafts' in the luminal leaflet of the membrane of the TGN. By interacting with the raft lipids, certain classes of apical proteins partition into the raft. By a mechanism that is presently not understood, rafts enter a transport vesicle or vacuole that targets to and fuses with the apical membrane. One experimental criterion to discern whether or not an epithelial membrane protein is sorted by raft-association is its insolubility in detergent at low temperature (287, 288). According to this definition, a raft pathway to the plasma membrane also exists in non-epithelial cells (287, 289). Moreover, the glycosphingolipid-raft pathway appears to be a specialized part of a general sphingolipid/cholesterol raft pathway between ER and plasma membrane (262). Sphingolipid rafts may first form in the cis/medial-Golgi at the site of sphenomelin synthesis. The enrichment of sphenolipids in the plasma membrane implies that rafts are transported towards trans-Golgi and TGN, while non-raft lipids like unsaturated phosphatidylcholine are selectively included in retrograde transport vesicles. This multistage refinement (262, 290) gradually concentrates the more saturated lipids in trans Golgi cisternae to a rigid sphingolipid/cholesterol remnant leaving the TGN. Interestingly, membrane proteins destined for the plasma membrane possess longer transmembrane helices than resident Golgi proteins supporting the notion that membrane transported to the cell surface is thicker than the Golgi membrane itself (268, 291). Increased thickness is typically expected for sphingolipid rafts (292).

Do GlcCer or higher glycosphingolipids function in the pathway to the melanosome by forming rafts in the TGN? This is unlikely: (I) Tyrosinase is not a typical raft protein since it is soluble in 1% TX-100 in the cold in both cell lines (not shown) and it is retained in the Golgi complex (or TGN) by its short transmembrane domain. It is released from the Golgi complex when its transmembrane domain is lengthened, probably because it now enters the thicker membrane (raft) destined for the plasma membrane, as is the case for TRP-1. (II) If rafts were the underlying principle for recruiting melanosomal membrane proteins into the AP-3 pathway, one would predict that tyrosinase and TRP-1 have transmembrane domains of similar length. This is evidently not the case. (III) Finally, a typical raft pathway exists from the TGN to the plasma membrane, and not the melanosome, in both MEB4 and GM95 cells as is exemplified by the detergent-insolubility and transport of a glycosylphosphatidylinositol-anchored protein (293).

Alternatively, GlcCer may play a role in recruiting tyrosinase and TRP-1 into a budding vesicle which involves binding of AP-3 to their cytosolic tails. GlcCer is synthesized on the cytosolic surface of the Golgi. We recently observed in fibroblasts that half of newly synthesized GlcCer is transported to the plasma membrane on the cytosolic surface of transport vesicles. Subsequently, it is removed from the cytosolic side by the multidrug transporter MDR1 P-glycoprotein (Raggers, R. et al., manuscript in preparation). An attractive scenario would be that GlcCer is involved in the recruitment of the cytosolic tails of melanosomal proteins by AP-3 in the TGN. After vesicle budding, GlcCer as a cofactor would be removed, and reattachment of the AP-3 adaptor complex prevented. Such a mechanism may be similar to the regulation of AP-1 and AP-2 activities by phosphoinositides (284, 294).

We observed that synthesis of GalCer (and galactosyldiglyceride) also restored tyrosinase transport (Figure 1). The galactolipids are not converted to higher glycolipids in GM95 and
MEB4 cells suggesting that monoglycosyl-lipids are the active species. Although GalCer is synthesized in the luminal leaflet of the ER membrane, experiments with short-chain GalCer have suggested that it has access to the same locations as GlcCer (122). If GalCer can substitute for GlcCer in the process of AP-3 mediated sorting and coat formation, this predicts the involvement of a cytosolic protein that recognizes both GlcCer and GalCer, like there is the glycolipid transfer protein (295). Alternatively, the protein-lipid interactions may be based on a physical phenomenon (like a glycolipid domain on the cytosolic surface).

The unexpected observation that glycosphingolipids have a structural function in a defined protein sorting step in the Golgi complex shines a new light on the role of glycosphingolipids in vesicular traffic. Our present findings indicate that some pigmentation defects notably in the class of the Hermansky-Pudlak syndrome may find their origin in aspects of glycosphingolipid metabolism. Such studies are now underway.

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Materials and methods

Materials

Material

Trans-[35S]label (>36 TBq/mmol) was from ICN (Costa Mesa, CA), D-erythro-[3-3H]sphingosine (0.65 TBq/mmol) from NEN Dupont (Boston, MA), and D-[1-14C]galactose and [1-14C]acetic acid (both 1.8 GBq/mmol) were from Amersham (Buckinghamshire, UK). Ceramides from bovine origin that contained both hydroxy- and non-hydroxy fatty acids were obtained from Matreya (Pleasant Gap, PA), whereas NBD-ceramide was from Molecular Probes (Eugene, OR). 4-methylumbelliferyl-β-N-acetylg glucosaminide and -β-galactoside were from Sigma (St. Louis, MO). M. Bornens (Institute Curie, Paris, France) kindly provided us with a mouse monoclonal antibody against CTR433. Rabbit antiserum against the cytoplasmic tail of tyrosinase (anti-pep7) and TRP-1 (anti-pep1) were kind gifts of V. Hearing (NIH, Bethesda, MD; 296). The rabbit polyclonal antibody A-14 against the human c-myc epitope was from Santa Cruz Biotechnology (Santa Cruz, CA) and the mouse monoclonal antibody 9E10 has been described previously (218). The mouse monoclonal TA99 was generously provided by K. Lloyd (Memorial Sloan-Kettering Cancer Center, NY; 297). Rabbit anticytathespisin D antiserum was a kind gift from K. von Figura (Göttingen, Germany; 273). Fluorescein-isothiocyanate (FITC)-labeled goat anti-rabbit and anti-mouse, and Texas red-labeled goat anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Goat anti-mouse antibodies coupled to horseradish peroxidase were from DAKO (Glostrup, Denmark). cDNA of mouse tyrosinase was kindly provided by F. Beermann (Swiss Inst. Exp. Cancer Res., Epalinges, Switzerland; 282), and cDNA of myc-tagged siaiytransferase by S. Munro (MRC, Cambridge, UK; 267, 268). N-butyldeoxyo-jirimycin was a kind gift of F. Platt (Department of Biochemistry, University of Oxford, UK; 265).

Plasmid construction
cDNA of CGlcT (205) was amplified in PCR reactions using CGlcT-pCDNA3 (chapter 3) as template and the following primer sets: for CGlcT, 5'-C GAGCTC GCC ATG GCG CTG CTG GAC CTG GCC-3' (forward) and 5'-C GAGCTC TTA TAC ATC TAG GAT TCC TCT CGC TG-3' (reverse); for CGlcT-KKVK with an ER retrieval signal at the C-terminus, 5'-C GAGCTC GCC ATG GCG CTG CTG GAC CTG GCC-3' (forward) and 5'-GC TCTAGA TTA
CTT GAC TTT CTT TAC ATC TAG GAT TTC CTC TGC TGT ACC-3' (reverse). PCR products were ligated into pCB7 cut with either *Sacl* or *SacI* and *Xbal*. GaIT-1 was released with *HindIII* and *Xbal* from GaIT-1-pCDNA3 (123) and inserted in pCB7 cut with the same enzymes. The cDNA of tyrosinase was released from tyrosinase-pCDNA/Amp (282) with *HindIII* and *Xbal* and inserted in pCB7. The putative transmembrane domain of tyrosinase was extended by ligating the oligonucleotide 5'-pGTAGTAATGCCCTG (PSI) in the *PstI* site of tyrosinase. This resulted in the incorporation of 6 hydrophobic amino acids, VLALVA, between A490 and A491 of tyrosinase. This construct is referred to as tyrosinase-TM6. To obtain a double myc-tag at the carboxy-terminus of tyrosinase and tyrosinase-TM6, both constructs were amplified in PCR reactions using tyrosinase-pCB7 and tyrosinase-TM6-pCB7 as templates for the first PCR and the following primers: 5'-CCA AAA TGT CGT AAT AAC CCC GCC CC-3' (forward) and 5'-GCC TCT AGA TCA AGA TAG TCT TCC CTC CAG TGT CGG CAT CGG CTG TTG CCA CAA GCT G-3' (reverse). The obtained PCR products served as templates for the second PCR using the same forward primer and 5'-GCC TCT AGA TCA AGA CAG GTC TCC CTC CGA GAT GAG CTG CGT CTC CAG TAG ATG CTT CAG CTT CAG CTA CAG CCT CTT CTC CAG ATG GCT CTG AT A CTA GCA A GC T G-3' as the reverse primer. PCR products were ligated into pCB7 cut with *HindIII* or *SacI* and *Xbal*. All constructs made by PCR were confirmed by sequencing both strands. cDNA of myc-tagged sialyltransferase was released with *HindIII* and *Xbal* from the original vector (267), and inserted in pCB7.

**Cell culture and transfection**

CGlcT-deficient GM95 cells and their parental MEB4 cells from the RIKEN Cell Bank (Tsukuba, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C with 5% CO₂. GM95 or MEB4 cells were transfected with the empty vector pCB7 (-mock; 257), or transfected with CGlcT-pCB7, CGlcT-KKVK-pCB7, GaIT-1-pCB7, myc-tagged sialyltransferase-pCB7, tyrosinase-pCB7, tyrosinase-TM6-pCB7, tyrosinase-myc-pCB7, or tyrosinase-TM6-myc-pCB7 using the calcium phosphate procedure (216). Transfectants were cultured in the presence of 200 U/ml hygromycin B. Stable cell lines were obtained by subcloning individual colonies. Expression was analyzed by measuring CGlcT or GaIT-1 enzyme activity as described (chapter 3). Expression of tyrosinase and tyrosinase-TM6 was assayed by Western blot using anti-pep7 antibody. Transfectants of myc-tagged sialyltransferase, tyrosinase-myc and tyrosinase-TM6-myc were screened with immunofluorescence microscopy using the 9E10 antibody. CHO cells were cultured as described (63). For all pigmentation experiments 1 mM D-tyrosine was included in the growth medium at the time of plating the cells.

**Cell fractionation**

GM95 cells in a 15 cm dish were swollen in hypotonic buffer, scraped and homogenized as described (122), except that 15 passes through a 25G5/8 needle were used. The postnuclear supernatant obtained by a 5 min 1,000g spin was loaded on top of a 0.7 - 1.5 M sucrose gradient and spun for 3 h at 265,000gmax. Enzyme activities were determined in 250 μl of each fraction using NBD-ceramide (122).

**Immunofluorescence microscopy**

Cells were grown on coverslips to 30-50% confluency. The cells were fixed with 3% paraformaldehyde and quenched in phosphate-buffered saline, containing 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺ (PBS) containing 50 mM NH₄Cl. Cells were then blocked and permeablitized for 1 h in PBS, 0.5% bovine serum albumin, 0.1% saponin (blocking buffer) and subsequently labeled with mixtures of primary antibodies in blocking buffer. The coverslips were washed for 45 min in blocking buffer with three buffer changes. Coverslips were incubated with 10% goat serum in blocking buffer for 20 min and subsequently counterstained for 30 min with fluorescently
labeled secondary goat antibodies at 1:50 dilutions in blocking buffer. The coverslips were then washed in blocking buffer for 45 min with three buffer changes, rinsed briefly in PBS and then water, and finally mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO). The cells were examined with a Leica confocal microscope (Leica, Heidelberg, Germany) using separate filters for each fluorochrome viewed (FITC: L_ex = 488 nm and L_em = 515 LP; Texas red: L_ex = 568 nm and L_em = 585 LP). Single-labeled cells with each primary/secondary antibody combination were examined, which showed that no bleed-through occurred for the given confocal conditions. Images were imported into Adobe Photoshop 4.0, and printed on a Tektronix dye sublimation printer. Before printing it was verified that every pixel in the image had a value between 1 and 255.

**Electron microscopy**

Cells were grown to subconfluency. In some samples, cells were incubated for 3 h at 37°C in medium containing 1 mM L-DOPA. The cells were fixed overnight at 4°C with 2% paraformaldehyde and 2.5% glutaraldehyde, post-fixed with 1% OsO_4, scraped and embedded in Epon. Finally, ultrathin sections were prepared, which were stained with 2% uranylacetate in distilled water for 45 min at 63°C.

**Melanin content**

Subconfluent cells on 10 cm dishes were used 3-5 days after seeding. For some experiments, cells were incubated for 3 h at 37°C in medium containing 1 mM L-DOPA. The cells were washed 3 times with ice-cold PBS, and gently scraped in PBS. A fraction of the cells was then used to determine the protein content using the BCA assay (Pierce, Rockford, IL). The remaining cells were pelleted at 1,000g, resuspended in 0.25 ml PBS, transferred to a 96-well microtiter plate and pelleted as above. To solubilize melanin, cell pellets were resuspended in 1 ml 1 M NaOH, vortexed vigorously and boiled for 30 min (298, 299). Samples were analyzed colorometrically, and pigmentation was expressed as A_475/mg protein.

**Tyrosinase activity**

L-DOPA oxidase activity of tyrosinase and TRP-1 was detected by zymography of SDS-PAGE gels. Samples were diluted without-reducing sample buffer and loaded directly on a 10% gel. After electrophoresis, the gels were incubated for 30 min at 37°C in 0.1 M phosphate buffer pH 6.8, containing 2 mM L-DOPA and 4 mM 3-methyl-2-benzothiazolinone hydrazone, as described (300).

**L-DOPA content**

Cells were grown for 3 days in 15 cm diameter dishes. Cell pellets were homogenized in 1 ml perchloric acid and pelleted. L-DOPA in the homogenates and in the culture media was determined by reverse-phase HPLC on a LC18 DB column (Supelco, Bellefonte, PA) using a mobile phase consisting of 0.1 M TCA (adjusted with sodiumacetate to pH 3.2) and electrochemical detection as described (301).

**Metabolic labeling of cellular lipids**

Subconfluent cells on 3-cm dishes were incubated with 1.5 ml culture medium containing D-[1-14C]galactose (37 kBq/ml), D-erythro-[3-3H]sphingosine (67 kBq/ml) or [1-14C]acetic acid (37 kBq/ml) in the presence or absence of drugs or lipid analogs for 48-72 h. Cells were washed three times with ice-cold PBS. Lipids were extracted, separated by thin layer chromatography, visualized by fluorography using X-ray films, scraped and quantitated, all as described (142).
Synthesis and transport of TRP-1 and tyrosinase

Expression of tyrosinase constructs was induced by 5 mM sodium butyrate (Fluka, Buchs, Germany) 14-16 h prior to experiment. Confluent cells on 3-cm dishes were washed twice with methionine- and cysteine-free Dulbecco's modified Eagle medium containing 20 mM Heps, pH 7.4 (pulse medium), incubated in pulse medium for 30 min at 37°C and labeled with 18 MBq/ml Tran[35S]label for either 15 or 60 min at 37°C. Cells were washed and chased in growth medium containing 5 mM methionine, 5 mM cysteine, and 20 mM Heps, pH 7.4 at 37°C. After different periods of chase time, the cells were cooled on ice and the remainder of the experiment was performed on ice or at 4°C. To assay cell surface delivery of newly synthesized proteins, cells were washed 3 times with PBS and incubated twice with PBS containing 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) for 20 min. Cells were washed twice with PBS, 10 mM glycine and incubated with PBS, 10 mM glycine for 20 min. Cells were lysed in PBS, 10 mM glycine, 0.5% v/v TX-100, 1 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl-fluoride and 1 µg/ml of the protease inhibitors aprotinin, chymostatin, leupeptin, and pepstatin A (lysis buffer with glycine) and centrifuged at 15,000g for 10 min. The supernatant was precleared during 1 h by incubation with protein A-Sepharose CL4B beads. Supernatant was subjected to immunoprecipitation using the TA99 antibody for TRP-1 or the A-14 antibody for tyrosinase-myc and tyrosinase-TM6-myc as described (219). Immunoprecipitates were resuspended in 50 µl elution buffer (150 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl pH 8.3, 0.5% w/v SDS, 1 mM phenylmethylsulfonyl-fluoride and 1 µg/ml protease inhibitors) and eluted during a 15 min incubation at 37°C. To quantitate the total amount of TRP-1, tyrosinase-myc, and tyrosinase-TM6-myc, 20% of each sample was saved for SDS-PAGE. The remainder of the supernatant was diluted 30-fold with wash buffer (150 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl pH 8.3, 0.1% w/v SDS, 0.5% w/v Nonidet P40, 0.5% w/v sodiumdeoxycholate, 1 mM phenylmethylsulfonyl-fluoride and 1 µg/ml protease inhibitors) adsorbed to immobilized streptavidin for 1 h to measure biotinylated TRP-1 or tyrosinase constructs. Beads were washed 4 times with wash buffer, and resuspended in 30 µl 20 mM Tris-HCl pH 6.8, 1 mM EDTA before addition of sample buffer, SDS-PAGE and phosphor imaging.

Antibody internalization

Cells in 3 cm dishes were incubated for 3 h at 37°C with 50 µg/ml anti-TRP-1 antibody TA99, or control antibody 9E10, in medium containing 20 µg/ml leupeptin. Cells were washed 5 times with ice-cold PBS and lysed in sample buffer. Equal amounts of protein were analyzed by SDS-PAGE and internalized antibody was detected by Western blotting with goat anti-mouse antibodies (anti-IgG) coupled to horseradish peroxidase. Reactivity of 9E10 with the anti-IgG was controlled by a Western blot of myc-tagged sialyltransferase. As an internal control, we also detected TRP-1 in the samples by Western blotting using the rabbit antibody anti-pep1. In parallel dishes containing cells grown on coverslips, we labeled internalized TA99 or 9E10 for immunofluorescence microscopy with FITC-labeled goat anti-mouse antibody.

β-galactosidase and β-hexosaminidase activity

Cells in 10 cm dishes were grown for 36 h as above, but with heat-inactivated fetal calf serum and with or without 5 mM mannos 6-phosphate. The media were removed and cells were lysed on ice. Cells and media were centrifuged at 20,000g for 20 min at 4°C to remove debris. All supernatants were stored at -80°C. The activities of β-galactosidase and β-hexosaminidase were measured in media and cell lysates, according to Galjaard (302) and Aerts et al. (303), respectively. Briefly, β-hexosaminidase activity was determined using 4-methylumbelliferyl-β-N-acetylglucosaminide as a substrate, in a 0.05 M/ 0.1 M citric acid/sodium phosphate buffer, pH 4.0. The β-galactosidase activity was measured in 0.1 M sodium acetate buffer, pH 4.3,
containing 100 mM NaCl, using 4-methylumbelliferyl-β-galactoside as a substrate. Enzyme activities were measured fluorometrically and were calculated from the rate of substrate hydrolysis ($L_{ex} = 366$ nm and $L_{em} = 445$ nm).

**Synthesis and secretion of cathepsin D**
Confluent cells were pulse-chased as above, with or without 5 mM mannose 6-phosphate in the chase medium. The media were then removed, the cells were washed twice with PBS and lysed in lysis buffer. After centrifugation of media and cell lysates at 20,000g for 20 min at 4°C, supernatants were precleared and subjected to immunoprecipitation, as described above, but with anti-cathepsin antiserum and analyzed by SDS-PAGE.

**SDS-PAGE and Western blot**
After the addition of 4x sample buffer (chapter 2), samples were heated for 5 min at 95°C, centrifuged briefly at 14,000g and resolved by SDS-PAGE on 10% minigels. Radiolabeled proteins were quantitated by phosphor imaging using Imagequant software. For Western blotting, polyvinylidene difluoride (PVDF) transfers were blocked for 90 min in PBS, 5% Protifar (Nutricia, Zoetermeer, The Netherlands), 0.2% Tween 20 (blotto). Primary antibody incubations were performed for 1 h in blotto. Detection was with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).