Inhibition of N-Glycan Processing in B16 Melanoma Cells Results in Inactivation of Tyrosinase but Does Not Prevent Its Transport to the Melanosome*

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Tyrosinase is the key enzyme in melanin biosynthesis, catalyzing multiple steps in this pathway. The mature glycoprotein is transported from the Golgi to the melanosome where melanin biosynthesis occurs. In this study, we have investigated the effects of inhibitors of N-glycan processing on the synthesis, transport, and catalytic activity of tyrosinase. When B16 mouse melanoma cells were cultured in the presence of N-butyldexynyojirimycin, an inhibitor of the endoplasmic reticulum-processing enzymes α-glucosidases I and II, the enzyme was synthesized and transported to the melanosome but almost completely lacked catalytic activity. The cells contained only 2% of the melanin found in untreated cells. Structural analysis of the N-glycans from N-butyldexynyojirimycin-treated B16 cells demonstrated that three oligosaccharide structures (Glc$_3$Man$_2$Glcnac$_2$) predominated. Removal of the glucose residues with α-glucosidases I and II failed to restore enzymatic activity, suggesting that the glucosylated N-glycans do not stericly interfere with the enzyme’s active sites. The mannosidase inhibitor deoxynojirimycin had no effect on catalytic activity suggesting that the retention of glucosylated N-glycans results in the inactivation of this enzyme. The retention of glucosylated N-glycans does not therefore result in misfolding and degradation of the glycoprotein, as the enzyme is transported to the melanosome, but may cause conformational changes in its catalytic domains.

N-Glycans can influence the properties of the protein to which they are conjugated in a number of different ways (1–3). In many cases N-glycans are required for protein folding, although there are examples where the addition of N-glycans to the protein is not required in order for the protein to fold (4). Each glycoprotein must therefore be studied on a case by case basis to determine what influence the N-glycan has on the biological and physical properties of a given glycoprotein. One strategy for studying the role(s) of N-glycosylation involves the use of specific inhibitors of N-linked oligosaccharide processing, such as α-glucosidase I inhibitors (5). In the presence of these compounds the nascent polypeptide is glycosylated co-translationally through the transfer of Glc$_3$Man$_2$Glcnac$_2$ from dolichol to the Asn residue of the Asn-X-Ser/Thr glycosylation sequon. However, the subsequent enzymatic processing steps, initiated in the ER by the action of α-glucosidase I, which removes the outer α-1,2-linked glucose residue from the oligosaccharide, followed by removal of the remaining two glucose residues in the ER by α-glucosidase II, are prevented (6). N-Glucosylated glycoproteins are therefore produced that lack hybrid and complex type oligosaccharide structures. The actions of inhibitors of α-glucosidases I and II, such as the imino sugar deoxynojirimycin (DNJ) and its N-alkylated derivatives, have been extensively characterized in cellular and viral systems (7). In the presence of these compounds, most N-glycans are arrested as glucosylated structures and undergo no further processing. However some glucosylated N-glycans are processed via the Golgi endomannosidase, even in cells treated with high concentrations of α-glucosidase inhibitors (8).

The correct folding of some glycoproteins is regulated within the ER through the interaction of the unfolded glycoprotein with calnexin and calreticulin, which have been shown to have chaperone-like functions (9, 10). Monoglucosylated, partially folded glycoproteins are bound by calnexin and retained in the ER until correct or complete folding takes place (11). Glycoproteins bound to calnexin are released through the action of α-glucosidase II and if they are still incompletely folded can be reglucosylated through the action of an ER-soluble glycosyltransferase, which only recognizes denatured domains on glycoproteins (4, 10). The re-glucosylated glycoprotein can then rebind to calnexin, and folding can continue. Calnexin binds poorly to glycoproteins carrying three untrimmed glucose residues, as it preferentially recognizes monoglucosylated species (11). Paradoxically, α-glucosidase inhibitors have minimal effects on cell viability and secretion (12), and cell lines deficient in α-glucosidases have a relatively normal phenotype (13). It therefore seems likely that alternative mechanisms exist within cells to enable the correct folding of many glycoproteins to occur, when they are prevented from interacting with their chaperones.

The effects of α-glucosidase inhibition on cellular glycoproteins are selective (14). For example, the transferrin receptor requires correct oligosaccharide processing for cell surface ex-
pression, whereas other cell surface glycoproteins in the same cell line are expressed normally (12). The common observation in mammalian systems is that some glucosylated glycoproteins fold normally (presumably those that are calnexin-independent or utilize other chaperones during their folding), whereas others fail to fold completely and are retained in the ER prior to degradation (15). As yet we have no insight into predicting the fate of a given glycoprotein when synthesized in a cell treated with inhibitors of α-glucosidase I. We also have little information concerning whether a glycoprotein can remain partially misfolded in one domain of the protein, as a result of retaining glucosylated N-glycans, while other domains adopt a relatively normal conformation and are recognized by the appropriate effector molecules within the cell.

The vast majority of studies to date have focused almost exclusively on the role of N-glycans on glycoproteins destined for secretion or cell surface expression. However, little information is available on the behavior of intracellular glycoproteins or glycosylated enzymes in α-glucosidase inhibited cells. In this study, we have investigated the function of an intracellular enzyme when it carries only glucosylated N-glycans by treating the cells with an inhibitor of α-glucosidase I. We have studied tyrosinase (monophenol, 3,4-B-dihydroxyphenylalaine, 3,4-DHPA) and its oxidizing activity (DOPA) in the presence of NB-DNJ, a specific inhibitor of N-glycosylation. From a total of six potential glycosylation sites, one has been implicated in the recognition of tyrosinase by calnexin and subsequent inhibition of melanogenesis. This site is located amino acid 90 (18). The enzyme was expressed in melanocytes, where it transits through the ER and Golgi and is transported to specialized intracellular organelles termed melanosomes (17). The enzymatic activity of tyrosinase is dependent upon the possession of two copper binding sites. At 37°C the reaction mixture was transferred to Whatman No. 3MM filter disks, washed with 0.1N HCl and then with acetone, dried, and scintillation counted. Melanin synthesis was expressed as picomoles of substrate utilized per hour/mg of protein.

Tyrosinase Hydroxylase Assay—The tyrosinase hydroxylase assay has been described previously and measures the [3H]l-tyrosine is hydroxylated to dopamine (16). Briefly, the reaction mixture contained 1 μmol DOPA and cell lysate or purified tyrosinase as a source of enzyme in 0.1 M sodium phosphate buffer, pH 7.2, in a total volume of 500 μl and incubated for 30 min at 37°C. The sample was then measured spectrophotometrically at 475 nm. One unit of tyrosinase was defined as the amount of enzyme catalyzing the oxidation of 1 mmol of DOPAchrome in 1 min at 37°C, using the molecular extinction coefficient of DOPAchrome at 475 nm as 3600 (23).

Melanin Assay—The melanin assay measures the production of acid-soluble melanin from l-[U-14C]tyrosine (100 μCi/ml, DuPont) and quantifies the entire reaction sequence, including tyrosinase and any post-tyrosinase catalytic activities and was performed according to Ref. 23. Briefly, the reaction mixture contained 1 μmol DOPA and cell lysate or purified tyrosinase as a source of enzyme in 0.1 M sodium phosphate buffer, pH 7.2, in a total volume of 500 μl and incubated for 30 min at 37°C. The reaction mixture was transferred to Whatman No. 3MM filter disks, washed with 0.1 N HCl and then with acetone, dried, and scintillation counted. Melanin synthesis was expressed as picomoles of tyrosine converted to melanin/mg of protein.

Isolation of Melanosomes—Enriched Fractions—Melanosomes were isolated from B16 and B16 NB-DNJ cells as described (24). Briefly, the cells were harvested, washed three times with PBS, and lysed with 10 mM phosphate buffer, pH 7.2, containing 0.25 M sucrose. A post-nuclear supernatant was obtained by centrifugation at 700 g for 10 min at 4°C and further fractionated by differential centrifugation at 11,500 x g for 30 min at 4°C, yielding a melanosome-enriched pellet.

Metabolic Labeling with [35S]Methionine/Cysteine—B16 cells were harvested, washed three times with PBS, and resuspended in methionine/cysteine-free RPMI 1640 medium (Life Technologies, Inc.). After 1 h, the reaction mixture contained 1 μmol DOPA and varying concentrations of cell lysate or purified tyrosinase as a source of enzyme in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose. A post-nuclear supernatant was obtained by centrifugation at 700 x g for 15 min at 4°C and further fractionated by differential centrifugation at 11,500 x g for 30 min at 4°C, yielding a melanosome-enriched pellet.
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containing 1% 2-mercaptoethanol and 0.9% SDS, for 48 h at 37 °C. Released oligosaccharides were desalted by passing the reaction mixture over a column of 100 μl each of Chelex 100, Dowex AG50 X-12 (H+ form), Dowex AG3 X-4A (OH− form). The eluate was pooled with a wash of 5 bed volumes of water, filtered, and concentrated before separation by gel permeation.

**Gel Permeation Chromatography**—Enzymatically released radiola-beled oligosaccharides were subjected to high resolution gel permeation chromatography using two Bio-Gel P-4 (1.5 × 100 cm) columns in series. The columns were maintained at 55 °C and eluted with water, and fractions (0.5 ml) were monitored for radioactivity using liquid scintillation counting. Elution positions in glucose units were determined by simultaneous separation of a ladder of partially hydrolyzed dextran and detected on the basis of refractive index.

**Electrophoresis and Western Blotting**—SDS-PAGE was performed as described by Laemmli (25) in 7.5% acrylamide gels. The samples were mixed 2:1 (v/v) with sample buffer (0.18 M Tris/HCl, pH 6.8, containing 15% glycerol, 9% SDS). To detect specific tyrosinase activity after electrophoresis, gels were incubated for 30 min at 37 °C with 0.1 M phosphate buffer, pH 6.8, containing 2 mm l-DOPA and 4 mm 3-methyl-2-benzothiazolinone hydrazone, as described previously (26). For Western blotting, cell or melanosomal lysates were separated by electrophoresis under denaturing conditions (sample was mixed 2:1 (v/v) with sample buffer containing 0.18 M Tris/HCl, pH 6.8, 15% glycerol, 9% SDS, 10% 2-mercaptoethanol and boiled for 5 min), transferred to Immobilon membrane (Amersham International, Amersham, UK) and reacted with αPEP7 antiserum (diluted 1:250). Visualization of antibodies was performed using the ECL chemiluminescent detection system (Amersham). Deyglycosylated samples for Western blotting were prepared by digestion with endog H or PNGase F. Digestion of crude extracts with endo H was performed following denaturation of samples in 0.15 M citrate buffer, pH 5.5, containing 2% SDS and 5% 2-mercaptoethanol and boiled for 5 min at 100 °C. Samples were cooled, diluted twice with water, 10 units/ml endo H (New England Biolabs) was added, and digestion performed for 24 h at 37 °C. For PNGase F digestion, samples were denatured by heating (5 min at 100 °C) in the presence of SDS reducing buffer (10 μl/100 μl of sample) and 1 unit/ml PNGase F added and incubated for 24 h at 37 °C.

**Immunocytochemistry**—B16 cells were harvested, washed in PBS, and resuspended at a density of 1 × 10^6/ml and 100 μl of the cell suspension spun onto a Cytospin slide (Cytospin, Shandon Scientific, Cheshire, UK). The slides were air-dried and acetone-fixed (30 s, ice-cold acetone), blocked in 5% FCS in PBS (30 min, room temperature in a humidified chamber), the blocking agent was removed, and the slides were incubated with the rabbit αPEP7 antiserum (1:100 in PBS/5% FCS, for 30 min at room temperature). The slides were washed three times in PBS and incubated as described above with anti-rabbit fluorescein isothiocyanate (Sigma). The slides were washed as described and mounted in Vectashield (Vector Laboratories, Burlington, CA) and examined by fluorescence microscopy (Zeiss, Axioplan).

**Confocal Microscopy**—B16 cells were cultured on sterile glass coverslips in individual wells of a 24-well plate (Costar) for 3 days in the presence or absence of 0.5 mM NB-DNJ. The cells were harvested, washed in PBS, fixed in ice-cold acetone for 30 s, air-dried, and immunostained as described for fluorescence microscopy (see above), except the secondary antibody used was anti-rabbit Texas Red. The cells were examined using a Bio-Rad MRC 1024 confocal microscope equipped with a 15-milliwatt cryoprotargon laser. The images were prepared using Lasersharp 2.1 and Adobe Photoshop 3.0. The scale bar represents 10 μm.

**Electron Microscopy**—Melanosome-enriched fractions were generated as described from B16 and B16 NB-DNJ, fixed in 4% glutaraldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.2, for 1 h at room temperature. The fractions were pelleted, washed three times with phosphate buffer, embedded in 4% agar, cut into 1-mm cubes, and fixed in 1% osmium tetroxide (Sigma). The samples were dehydrated through an ethanol series followed by propylene oxide and embedded in Epon 812 (Sigma). The sections were stained with uranyl acetate/lead citrate and examined with a Tesla-500 electron microscope.

**RESULTS**

**Tyrosinase Activity and Melanin Biosynthesis**—B16 cells were cultured in the presence (B16 NB-DNJ) or absence (B16) of 50 μM or 500 μM NB-DNJ for 3 days. When the cells were visually inspected the untreated cells exhibited normal melanin pigmentation, whereas 500 μM NB-DNJ treatment resulted in nonpigmented cells. B16 cells treated with 50 μM NB-DNJ exhibited an intermediate degree of melanin content (Fig. 1A). When the two tyrosinase isoenzyme activities were visualized on gels following electrophoresis of crude extracts derived from NB-DNJ treated or untreated cells, tyrosinase activity could only be detected in extracts from untreated cells (Fig. 1B). Both activities of tyrosinase (tyrosine hydroxylase and DOPA oxidase) were profoundly inhibited by NB-DNJ treatment, and this inhibition was dose-dependent (Fig. 2A). When cells were treated with 500 μM NB-DNJ, tyrosinase hydroxylase, activity decreased by 85%, DOPA oxidase activity by 95%, and melanin content decreases by 98% relative to untreated cells (Fig. 2A). The inhibition of tyrosinase activity and melanin biosynthesis was observed within 24 h following NB-DNJ treatment (Fig. 2B). Total reversibility of tyrosinase activity and melanin biosynthesis was observed when NB-DNJ was washed out of the cultures (Fig. 2C). The effect of NB-DNJ on tyrosinase activity was found to be indirect. This was demonstrated by incubating untreated cells lysates with NB-DNJ (0.5 mM) for 2 h prior to the DOPA oxidase assay. No significant inhibition of tyrosinase activity was observed (Table I). The specific activity of tyrosinase was found to correlate with treatment of B16 cells with known inhibitors of α-glucosidases I and II such as castanospermine, DNJ, and N-methyl-DNJ (data not shown), whereas the mannosidase inhibitor DMJ failed to inhibit tyrosinase activity and did not inhibit melanin biosynthesis (Table I). The specific activity of tyrosinase was found to be very similar in the total cell lysate from B16 cells and the melanosomal fractions (Table I).

**Synthesis and Transport of Tyrosinase**—Cells were metabolically labeled with [35S]methionine/cysteine for 4 h and immunoprecipitated with anti-αPEP7 antiserum. A 72-kDa species was precipitated from B16 cells and a 69-kDa species from B16 NB-DNJ cells (Fig. 3A). Based on cell lysates obtained from the same number of B16 and B16 NB-DNJ cells, the level of B16 NB-DNJ tyrosinase detected by immunoprecipitation was unchanged by NB-DNJ treatment. To determine whether or not the tyrosinase detected in whole cell lysates from B16 NB-DNJ...
cells was localized to the melanosome, a melanosome-enriched fraction was generated by differential centrifugation (24). The pellet containing the melanosomes from untreated cells was darkly pigmented (black) while the supernatant remained clear indicating that the pellet contained melanosomes. The same fraction from NB-DNJ-treated cells was lightly pigmented reflecting the loss of melanin resulting from drug treatment. This was further confirmed by electron microscopy on the melanosomal enriched fraction (Fig. 3B). As anticipated this fraction contained melanosomes at various stages of development, as indicated by their degree of pigmentation and their heterogeneous size. The fraction from B16<sup>−NB-DNJ</sup> contained organelles of comparable size to those from untreated cells, but they were less electron-dense, indicating reduced levels of melanin. Similar results to those observed with whole cell lysates were obtained by immunoprecipitating tyrosinase from the melanosomal enriched fraction of B16 and B16<sup>−NB-DNJ</sup> cells (Fig. 3A), indicating the presence of the enzyme in the melanosome. Tyrosinase expression was further assessed by Western blotting. As presented in Fig. 4A, identification of tyrosinase from melanosomal extracts with anti-tyrosinase antibodies showed the same decrease in B16<sup>−NB-DNJ</sup> tyrosinase molecular weight compared with the B16 enzyme in the immunoprecipitation experiments. Treatment of B16 and B16<sup>−NB-DNJ</sup> tyrosinase with PNGase F, which hydrolyzes the β-aspartylglycosylamine bond between Asn and the innermost GlcNAc of the N-glycan, yielded a 60-kDa polypeptide for both enzymes (Fig. 4A). PNGase F digestion showed that the B16<sup>−NB-DNJ</sup> tyrosinase was synthesized as a 60-kDa polypeptide chain and co-/post-translationally modified to give the mature 69-kDa glycoprotein. Moreover, extraction of melanosomal membrane proteins with Triton X-114 followed by Western blotting demonstrated that B16<sup>−NB-DNJ</sup> tyrosinase was localized to the melanosomal membrane (Fig. 4B). The same localization was revealed for B16 tyrosinase (Fig. 4B), in good agreement with previous reports demonstrating that tyrosinase is an integral membrane glycoprotein (27). Localization of the enzyme was confirmed by immunocytochemistry (Fig. 5), showing that there was no qualitative or quantitative difference in the general location of tyrosinase in B16 and B16<sup>−NB-DNJ</sup> cells. We examined the distribution of tyrosinase at higher resolution by confocal microscopy (Fig. 6) and observed a punctate cytoplasmic pattern of staining in both B16 and B16<sup>−NB-DNJ</sup> cells, confirming the melanosomal localization of tyrosinase, irrespective of NB-DNJ treatment.

**Structural Analysis of B16<sup>−NB-DNJ</sup> Tyrosinase Oligosaccharides**—Incubation of B16 and B16<sup>−NB-DNJ</sup> tyrosinases with endo H resulted in changes in the electrophoretic mobilities of both glycoproteins. While B16<sup>−NB-DNJ</sup> tyrosinase was completely sensitive to endo H, B16 tyrosinase was only partially sensitive, suggesting that tyrosinase normally contains both high mannose and complex type oligosaccharides (Fig. 4A). Increased endo H sensitivity was also observed with tyrosinase derived from DMJ-treated cells (Fig. 4A). The status of the tyrosinase N-glycans from B16 and B16<sup>−NB-DNJ</sup> cells was investigated further by labeling the cells with [2-<sup>3H</sup>]mannose for 24 h and B16 and B16<sup>−NB-DNJ</sup> tyrosinases isolated from the cell lysates as described. Release of [2-<sup>3H</sup>]mannose-labeled unreduced oligosaccharides from the purified enzymes was achieved absence of 0.5 mM NB-DNJ and cell lysates analyzed for enzymatic activity and melanin content. C, cells were incubated for 72 h in the presence or absence of different concentrations of NB-DNJ and cell lysates analyzed for enzymatic activity and melanin content. B, cells were incubated for 72 h in the presence or absence of different concentrations of NB-DNJ and cell lysates analyzed for enzymatic activity and melanin content.
DOPA oxidase activity of tyrosinase was assayed as described under “Experimental Procedures.” Results are presented as the means of triplicate determinations (the standard deviations fell within ±10%, data not shown). B16 + NB-DNJ indicates cells pretreated for 3 days with 0.5 mM NB-DNJ. B16 + NB-DNJ indicates that a B16 cell lysate was incubated for 2 h with 0.5 mM NB-DNJ prior to DOPA oxidase assay. B16 + DMJ indicates cells pretreated for 3 days with 0.5 mM DMJ. Purified tyrosinase from B16 and B16 + NB-DNJ were digested with a mixture of α-glucosidases I and II for 24 h at 37 °C. ND, not determined.

| Sample                      | B16        | B16 + NB-DNJ | B16 + NB-DNJ | B16 + DMJ |
|-----------------------------|------------|--------------|--------------|-----------|
| Cell lysate                 | 1.5        | 0.075        | 1.5          | 1.8       |
| Melanosome lysate           | 1.5        | 0.074        | ND           | 1.8       |
| Microsome lysate            | 1.3        | 0.074        | ND           | 1.6       |
| Purified tyrosinase         | 1200       | 59.5         | ND           | ND        |
| Purified tyrosinase + α-glucosidases I and II | 1200       | 59.4         | ND           | ND        |

Fig. 3. A, immunoprecipitation of tyrosinase. B16 cells were labeled with [35S]methionine/cysteine for 4 h in the presence or absence of 0.5 mM NB-DNJ. Cell lysates and melanosomal lysates were immunoprecipitated with αPEP7 antiserum and visualized by autoradiography. B, ultrastructure of melanosome-enriched fraction. Electron microscopy on the melanosome-enriched fraction from B16 and B16 + NB-DNJ cells.

Fig. 4. Western blotting of B16 and B16 + α-glucosidases. A melanosomal lysate obtained from 10^4 cells (treated with imino sugars as indicated) was treated or untreated with PNGase F (0.1 unit/ml) or endo H (10 units/ml), separated by 7.5% SDS-PAGE, Western blotted, and the tyrosinase detected immunologically with anti-tyrosinase antiserum (αPEP7). B, melanosomal membrane proteins were extracted with Triton X-114 and analyzed as described for A.

Fig. 5. Intracellular immunolocalization of tyrosinase by immunofluorescence microscopy. B16 (A) and B16 + NB-DNJ (B) cells were stained with αPEP7 antiserum and detected with anti-rabbit fluorescein isothiocyanate. They were examined by fluorescent microscopy.

Table I
Tyrosinase activity in treated and untreated melanoma cells

| Sample                      | B16        | B16 + NB-DNJ | B16 + NB-DNJ | B16 + DMJ |
|-----------------------------|------------|--------------|--------------|-----------|
| Cell lysate                 | 1.5        | 0.075        | 1.5          | 1.8       |
| Melanosome lysate           | 1.5        | 0.074        | ND           | 1.8       |
| Microsome lysate            | 1.3        | 0.074        | ND           | 1.6       |
| Purified tyrosinase         | 1200       | 59.5         | ND           | ND        |
| Purified tyrosinase + α-glucosidases I and II | 1200       | 59.4         | ND           | ND        |
cose residues had been removed. The tyrosinase post-glucosidase digestion was always observed to be more immunoreactive with the anti-tyrosinase antibody on Western blots, suggesting that there was increased exposure of the C-terminal epitope recognized by this antiserum following de-glucosylation (Fig. 10). As shown in Table I, the deglucosylated B16<sup>−</sup>NB-DNJ tyrosinase showed the same enzymatic activity as glucosylated B16<sup>+</sup>NB-DNJ tyrosinase.

**DISCUSSION**

In this report we have examined the effects of inhibiting N-glycan processing, using the α-glucosidase inhibitor NB-DNJ, on the biological properties of murine tyrosinase. We have found that, in contrast to some other glucosylated glycoproteins (12, 15), the enzyme does not misfold in the presence of glucosylated N-glycans to a degree that causes it to be retained in the ER and degraded. Instead it is transported to its correct cellular location, namely the melanosome. Indeed, the amounts of tyrosinase immunoprecipitated from the melanosomes of B16 and B16<sup>−</sup>NB-DNJ cells are comparable, showing that similar levels of B16 and B16<sup>−</sup>NB-DNJ tyrosinase are synthesized and transported to the correct organelle, irrespective of their N-glycan composition. This implies that either the folding of the protein in regions required for transport must be comparable with the native molecule or that the transport is independent of its folding. However, despite correct localization this enzyme has virtually no catalytic activity in B16<sup>−</sup>NB-DNJ cells, and therefore the cells cannot support melanogenesis, and they are profoundly deficient in pigment relative to B16 cells.

Tyrosinase is a bifunctional enzyme in that it catalyzes the hydroxylation of tyrosine to DOPA (the rate-limiting step in melanin biosynthesis) and the oxidation of DOPA to DOPA quinone. For the first step, tyrosinase requires DOPA as a co-factor, and the enzyme has been proposed to contain two catalytic sites involved in the binding of tyrosine and DOPA, respectively (16). Tyrosine hydroxylase activity in NB-DNJ-treated B16 cells is diminished to 15% of its normal activity and DOPA oxidase activity to 5%, indicating that both catalytic sites have been affected. Upon removal of NB-DNJ from the culture medium the cells re-synthesize tyrosinase, and therefore both activities (tyrosine hydroxylase and DOPA oxidase) of the enzyme can be detected in cell extracts within 24–72 h. Tyrosinase dysfunction in B16<sup>−</sup>NB-DNJ cells is also indicated by the marked de-pigmentation of these cells due to the incapacity
of the B16<sup>NB-DNJ</sup> tyrosinase to initiate melanin biosynthesis. Conversely, others have reported that the overall activity of mouse tyrosinase is not inhibited when B16 cells are incubated (for 11–25 days) with glucosamine, a weak glucosidase inhibitor, despite the loss of pigmentation (28, 29). These results, obtained by monitoring enzyme activity, were interpreted as an indication that some isoforms of tyrosinase fail to reach the melanosome. In contrast, immunoprecipitation and Western blotting experiments, performed in this study, have shown that B16<sup>NB-DNJ</sup> tyrosinase is correctly transported to the melanosomes. Further support comes from the report of the effects of α-glucosidase inhibitors on human tyrosinase (30). The enzyme was still synthesized in the human cell line MM96E, but had no activity and therefore the cells were nonpigmented (30). This is therefore analogous to our findings with NB-DNJ-treated B16 mouse melanoma cells. Moreover, Triton X-114 extraction of the B16<sup>NB-DNJ</sup> cells demonstrated that B16<sup>NB-DNJ</sup> tyrosinase is correctly incorporated in the melanosomal membrane in an analogous fashion to the B16 tyrosinase. For melanogenesis this is an important observation, as it is known that even if the enzyme is active immediately after completion of its synthesis, melanin synthesis is only initiated after tyrosinase reaches the melanosomal membrane (27). Correct insertion of B16<sup>NB-DNJ</sup> tyrosinase into the membrane suggests that the de-pigmentation observed in B16<sup>−</sup> cells is due to inactivity of tyrosinase, rather than its incorrect transport. In addition, the same low level of activity of B16<sup>NB-DNJ</sup> tyrosinase was found both in melanosomal and microsomal fractions, suggesting that inactivation does not occur during, or as a consequence of, defective transport. On the other hand, direct incubation of untreated cell lysates with NB-DNJ results in the detection of normal levels of tyrosinase activity. This shows that NB-DNJ does not act as a direct inhibitor of this enzyme but that it causes an indirect effect on tyrosinase activity, presumably through the alteration in N-glycan structure. We conclude that B16<sup>NB-DNJ</sup> tyrosinase is therefore synthesized in an inactive form. Digestion with PNGase F revealed that B16<sup>NB-DNJ</sup> tyrosinase is synthesized as a 60-kDa polypeptide. The same molecular weight was obtained for PNGase-treated B16 tyrosinase. This is in agreement with the predicted polypeptide molecular weight based on the tyrosinase cDNA, which predicts 533 amino acids, corresponding to a molecular mass of 58–60 kDa (19). Following co- and post-translational attachment of carbohydrates, the molecular mass of B16 tyrosinase increases to approximately 72 kDa (19). Mature B16<sup>NB-DNJ</sup> tyrosinase has a molecular mass of only 69 kDa, 3 kDa less than B16 tyrosinase. This indicates that the differences in molecular mass appear to be at the post-translational level and are related to differences in the oligosaccharide moiety.

Analysis of B16<sup>NB-DNJ</sup> tyrosinase oligosaccharide sequences revealed the existence of glucosylated oligosaccharides, including Glc<sub>3</sub>Man<sub>9</sub>, Glc<sub>3</sub>Man<sub>8</sub>, and Glc<sub>3</sub>Man<sub>7</sub>, which is in agreement with the N-glycan structures found on gp120 expressed in the presence of NB-DNJ (31). The small percentage of more processed N-glycan structures observed could explain the low level of residual tyrosinase activity observed in this study and may result from the action of the Golgi endomannosidase (8). Our results show that the untreated tyrosinase has a mixture of high mannose and complex type oligosaccharides. Similar N-glycan profiles have been reported for hamster tyrosinase (23), suggesting that there is a degree of conservation of tyrosinase N-glycan structures among at least two mammalian species.

To determine whether the lack of complex N-glycans was sufficient to render tyrosinase inactive, B16 cells were treated with DMJ, an inhibitor of Golgi α-1,2-mannosidase. Tyrosinase was rendered partially sensitive to endo H following treatment of B16 cells with DMJ. However, there was no effect on tyrosinase activity, suggesting that the absence of complex type N-glycan structures is not responsible for the maintenance of tyrosinase in an active form. Furthermore, these data suggest that retention of glucosylated high mannose structures, due to NB-DNJ treatment, directly results in the loss of tyrosinase activity. This is not due to the glucosylated N-glycan directly interfering with the catalytic site as was shown by removing the three glucose residues, which did not restore enzyme activity. The loss of activity therefore may result from partial misfolding of the catalytic site, possibly showing that folding of this domain is chaperone dependent. It is interesting to note that the transport to the melanosome remains normal. Perhaps this illustrates that the overall conformation of this protein occurs by independent domain folding mechanisms.

Similar conclusions have been drawn from studies of human immunodeficiency virus glycoproteins expressed in NB-DNJ-treated Chinese hamster ovary cells (32). The structure of gp120 was probed using a panel of over 40 monoclonal antibodies. It was found that most of the regions of gp120 expressed in the presence of NB-DNJ (gp120<sup>−</sup>) were indistinguishable from gp120 expressed in the absence of the compound (gp120<sup>−</sup>). Furthermore, the gp120<sup>−</sup> retained its ability to bind to CD4 with an affinity comparable with gp120<sup>−</sup>. However, when the conformation of the V1/V2 loops were investigated, it was found that this region was affected by the retention of glucosylated N-glycans. This alteration in antibody recognition was attributed to a change in the conformation of this region of the molecule. It is of interest that gp120<sup>−</sup> transits through the ER and Golgi and is secreted from the cell at comparable rates relative to gp120<sup>−</sup>, yet has localized conformational changes due to retention of glucosylated N-glycans during protein folding (32). We conclude that the retention of glucosylated N-glycans results in the loss of catalytic activity but does not impair intracellular transport. As a consequence, melanogenesis is blocked due to the pivotal nature of this enzyme in the melanin biosynthetic pathway. Inactivation of tyrosinase may result from the failure of the enzyme to bind copper either through changes in the conformation of the active site in the presence of glucosylated N-glycans or indirectly due to impaired copper transport within the cell. Another possibility is
that the protein undergoes independent domain folding with the active site conformation being critically dependent on the interaction of the protein with chaperones during its folding. The mechanism(s) of tyrosinase inactivation in NB-DNJ-treated cells is currently under investigation.

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