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Functional Analysis of Tyrosinase Isozymes of Cultured Malignant Melanoma Cells During the Recovery Period Following Interrupted Melanogenesis Induced by Glycosylation Inhibitors*

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Multiple forms of tyrosinase, T_1, T_2, T_3, have been shown to differ with respect to carbohydrate moieties of these isozymes. We demonstrated that, in cultured B-16 melanoma cells, melanization can be completely interrupted by glycosylation inhibitors, such as glucosamine and tunicamycin, and that these inhibitors cause a selective loss of membrane-bound T_3. It is further found that inhibition of melanization induced by glucosamine occurs even in the presence of protease inhibitors, such as phenylmethylsulfonyl fluoride and leupeptin, and that melanization inhibition is reversible upon removal of the inhibitor. In this report we have also examined the process of development and recovery of the tyrosinase isozymes in cells in which the interruption of melanogenesis has been released by the removal of these glycosylation inhibitors. The recovery process, which occurs during the period after interrupted melanogenesis and is a process of remelanization, has been biochemically followed. Tyrosinases obtained from the deoxycholate-solubilized large-granule fraction of these melanoma cells have been analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immediately after removal (0 h recovery) of the glycosylation inhibitor, loss of melanization and T_3 is accompanied by T_1 heterogeneity which is visualized as two electrophoretically distinct species, T_1' and T_1''. At this time, T_1' and T_1'' do not have a concanavalin A affinitive carbohydrate moiety but do possess in vitro dopa reactivity. When recovery of melanization begins visibly 24 h later, T_3 is re-formed with disappearance of T_1 heterogeneity. By 48 h, the previous normal level of melanization is almost attained. These results suggest that maturation of tyrosinase may occur via T_1' and T_1'' as precursors of T_3, or possibly T_1 through the addition of N-glycosydically linked oligosaccharide moieties which can be interrupted by glucosamine and tunicamycin.

Recent research has considerably increased our knowledge of the regulatory mechanism of eumelanogenesis [1–5]. One of our current interests has been the intracellular maturation of tyrosinases which is found to be closely related to their N-glycosydically linked oligosaccharides [6,7]. Tyrosinase has been reported to contain carbohydrate moieties such as sialic acid [8], mannose, and galactose [9], and maturation of tyrosinase seems to proceed from T_2 to T_1 and further to T_3 with sequential changes in their carbohydrate moieties as tyrosinase moves from ribosome to premelanosomes through such melanogenic smooth membrane compartments as Golgi-associated endoplasmic reticulum (GERL) and coated vesicles [2].

In contrast to the previous concept of early melanogenesis such as melanogenically active tyrosinases and premelanosome formation, each originating in a single functional segment, the Golgi apparatus [10], we have found that tyrosinase synthesized by ribosomes becomes active through protein glycosylation and processing in the GERL and coated vesicles which functionally and structurally are different units from the Golgi apparatus [2,3]. Further, the inhibition of such protein of glycosylation in cultured melanoma cells using glucosamine [11–14] and tunicamycin [15–17] has been found to result in the depigmentation of these cells accompanied by the loss of T_3 tyrosinase and premelanosome alteration [6,7].

In this paper, we have made a functional analysis of tyrosinase isozymes by studying changes that lead to and are necessary for the recovery of melanin synthesis after removal of glycosylation inhibition with the special aim of clarifying initial melanogenesis.

MATERIALS AND METHODS

Materials

Tunicamycin was obtained from Dr. Tamura of the Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan, through Dr. Kobata of the Department of Biochemistry, University of Kobe, Kobe, Japan. [^{3}H]Mannose(D-[2-^{3}H(N)]mannose, 10–20 Ci/mmol) and [^{14}C]tyrosine(L-[^{14}C(U)]tyrosine, 450 mCi/mmol) were purchased from New England Nuclear (Boston, Massachusetts). Glucosamine hydrochloride, dihydroxyphenylalanine, cycloheximide, and all other materials were obtained from Sigma Chemical Co (St. Louis, Missouri).

Cells

B-16 mouse melanotic melanoma cells were cultured in Eagle's minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C with 5% CO_2/95% air atmosphere. Glucosamine hydrochloride added at the concentration of 1 mg/ml (86.5% inhibition of [^{3}H]mannose uptake [6]) culture medium was used immediately after cell seeding. Tunicamycin experiments were carried out 24 h after cell seeding using the culture medium containing 0.2 ng/ml of tunicamycin (69.8% inhibition of [^{3}H]mannose uptake [6]). Thereafter all medium was replaced with the inhibitor-containing fresh medium twice a week during the cell growth for present experiments. Removal of glycosylation inhibition was carried out after exposure of these cells to the inhibitors for 10–25 culture days, inducing the complete loss of their melanization. Thus, induced unmelanized melanoma cells were washed 3 times with Hanks' buffer and recultured with fresh medium free of glycosylation inhibitors for subsequent biochemical examinations.

Effect of Protease Inhibitors: For the effect of protease inhibitors on glycosylation-induced depigmentation, phenylmethylsulfonyl fluoride (PMSF) (0.1–5 mM) or leupeptin (LPT) (0.1 mM) was added to the present culture system together with glycosylation inhibitors, and

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Abbreviations:
DOC: deoxycholate
GERL: Golgi-associated endoplasmic reticulum
LGF: large-granule fraction
LPT: leupeptin
PB: phosphate buffer
PMSF: phenylmethylsulfonyl fluoride
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA: trichloroacetic acid

culture was carried to a confluency. The degree of pigmentation was observed after pelleting the cells.

Effect of Cycloheximide: Cycloheximide was added at the concentration of 0.05 ng/ml immediately following removal of glycosylation inhibitors.

Incorporation Experiment

For the examination of the effect of drugs on the synthesis of carbohydrates, cells were cultured in Falcon plastic culture dishes (35-mm diameter) containing three 15 mm-diameter glass coverslips per dish and then 1 μCi per ml of [^{3}H]mannose was added to the cultures for a desired period of time. After incubation, the cells were washed 3 times with cold Hanks' balance solution and 5% cold trichloroacetic acid (TCA) was added twice every 10 min, followed by successive treatments with 70%, 90%, and 100% EtOH to dry the cells. The TCA-insoluble fraction was dissolved in 0.5 ml of Soluene and mixed with 10 ml of toluene-based scintillation fluid. The radioactivity was counted in a liquid scintillation spectrometer. Melanogenic activity was assayed using incorporation of [^{14}C]tyrosine (10 μCi/ml) in the presence of 5 ng/ml of cycloheximide in the same manner. A cell count was done using a hemocytometer after trypsinization.

Fractionation

Subcellular fractionation was performed by the method of Seiji et al [10] using the cell homogenate from about 40 falcon culture dishes (75 cm^2). Thus, the cells collected after trypsinization were homogenized in ice-cold 0.25 M sucrose solution containing 1 mM PMSF using a Teflon homogenizer chilled with ice. The suspension obtained was first centrifuged at 700 *g* for 10 min to remove unruptured cells and nuclear debris. The residue was discarded. The supernatant was further centrifuged at 12,000 *g* for 10 min to obtain a soluble fraction and the large-granule fraction (LGF) as a sediment.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The LGFs from the cell homogenate were suspended in 0.5% sodium deoxycholate (DOC) aqueous solution containing 1 mM PMSF for 20 min at 4°C and were centrifuged at 105,000 *g* for 60 min. The DOC-solubilized LGF was mixed with an equal volume of buffer A (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.004% of bromphenol blue). The SDS gel electrophoresis was carried out at pH 8.7 in Tris-glycine buffer containing 1% SDS at 1 mA per gel according to the method of Davis [18]. After electrophoresis, the gel was washed with 0.3 M phosphate buffer (PB) and then incubated in 0.1% dopa in 0.1 M PB (pH 6.8) at 37°C for 5 h.

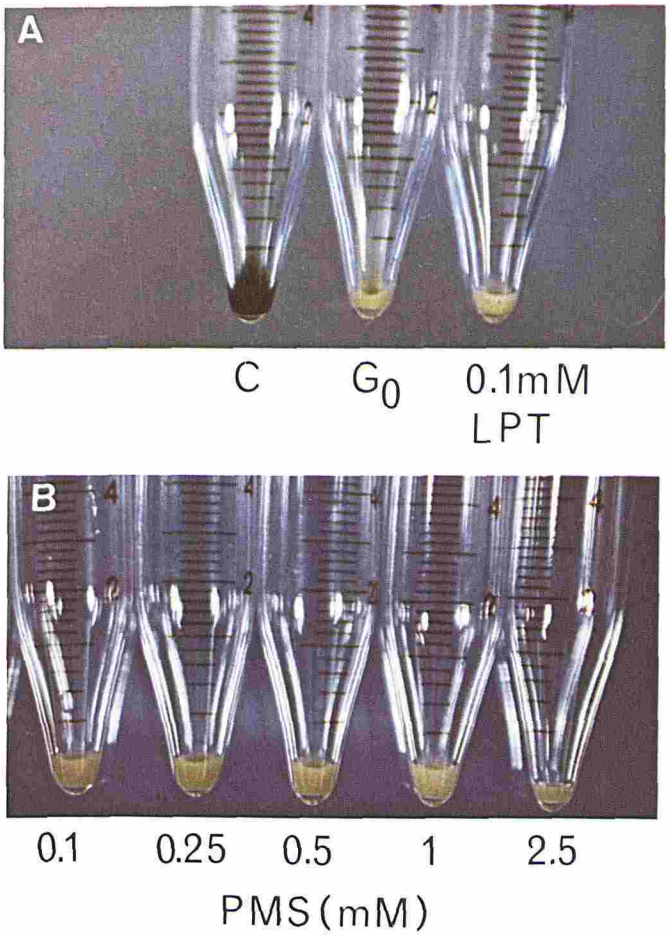

FIG 1. Effect of protease inhibitors, LPT and PMSF on the Glc-induced depigmentation of cultured melanoma cells. The cells were cultured with glucosamine in the presence of 0.1–2.5 mM PMSF or 0.1 mM LPT for 10 days. *A*, Control, non-treated B-16 cells, G_0, Glc-treated cell, Lpt, Glc + LPT treated cell, *B*, PMS, Glc + PMSF treated cell.

RESULTS

Effect of Protease Inhibitors

In an attempt to check the possibility that the depigmenting effect by glycosylation inhibition is a secondary event due to the enhanced activity of cellular proteases, the effect of protease inhibitors such as PMSF (0.1–2.5 mM) and LPT (0.1 mM) on glucosamine-induced depigmentation of B-16 melanoma cells was examined after pelleting the cells that had reached confluency in the presence of these protease inhibitors. The addition of protease inhibitors did not apparently inhibit the depigmenting effect by glucosamine even at a concentration in which the protease inhibitors partially inhibit the cell growth (Fig 1).

Gross and Light Microscopic Observations

The recovery process from interrupted melanogenesis induced by the glycosylation inhibitors, glucosamine (1 mg/ml) and tunicamycin (0.2 ng/ml), can be clearly visualized after pelleting the cells. Following the induction of pigment loss, the addition of fresh medium without glycosylation inhibitors induces reappearance of melanin pigment in the cells (Fig 2). This recovery process of melanization begins visibly 2 days after removal of the inhibitors. By 3 or 4 days, the pre-interrupted level of melanization is achieved with glucosamine or tunicamycin-treated cells, respectively. Phase contrast microscopic observations of the cells that have lost and then recovered the pigmentation, indicate that there are no substantial morphologic changes of the cells with the exception of the amounts of melanin pigment during the recovery period (Fig 3).

Electrophoresis

In SDS-PAGE patterns of the DOC-solubilized LGF from glucosamine-treated cells, membrane-bound T_3 tyrosinase is lost and T_1 tyrosinase appears to be resolved into additional electrophoretic- and molecular weight-distinct components, T_1' and T_1''. These changes in enzyme pattern occur in glucosamine-treated cultures even in the presence of protease inhibitors such as PMSF and LPT (Fig 4). By further illustration, the time-dependent sequence and pattern for this enzyme development is shown in Fig 5. Within 1 day after removal of the glycosylation inhibitor, glucosamine, new melanin formation is not visible. However, the appearance of T_1' and T_1'' tyrosinases has almost disappeared. Two days after removal of the inhibitor, the ratio of T_3 to T_1 tyrosinase appears to resemble that of the control, and melanin pigmentation has been visibly restored (Figs 5*A*,*B*, 2*A*, respectively). For tunicamycin-treated cells, this pattern of T_3 reappearance, T_1' and T_1'' disappearance, and visualization of melanin is slightly delayed (Figs 5*C*,*D*, 2*B*, respectively).

Cycloheximide Effect

In order to evaluate the effect of inhibiting protein synthesis during the period of recovery of melanogenesis, following gly-

cosylation inhibition, 0.05 ng/ml of cycloheximide was added to the recovery system. This concentration of cycloheximide causes a 45% inhibition of protein synthesis [6]. Our results demonstrated that in spite of a 45% inhibition of protein synthesis after the removal of glucosamine, visible melanization occurs, in a manner similar to that in the absence of cycloheximide, within 2 days (Fig 6*A*). SDS-PAGE (Fig 6*B,C*) shows that, similar to the recovery pattern present in Fig 5 in which a specific inhibitor of protein synthesis was not added, T_3 tyrosinase is clearly re-formed with a disappearance of newly induced T_1 tyrosinases by 1 day after removal of the glycosylation inhibitor. Furthermore, Fig 6*D* shows that after the release from glycosylation inhibition and in the presence of cycloheximide, the uptake of [^{14}C]tyrosine increases concurrently with the recovery of protein glycosylation as revealed by the uptake of [^{3}H]mannose.

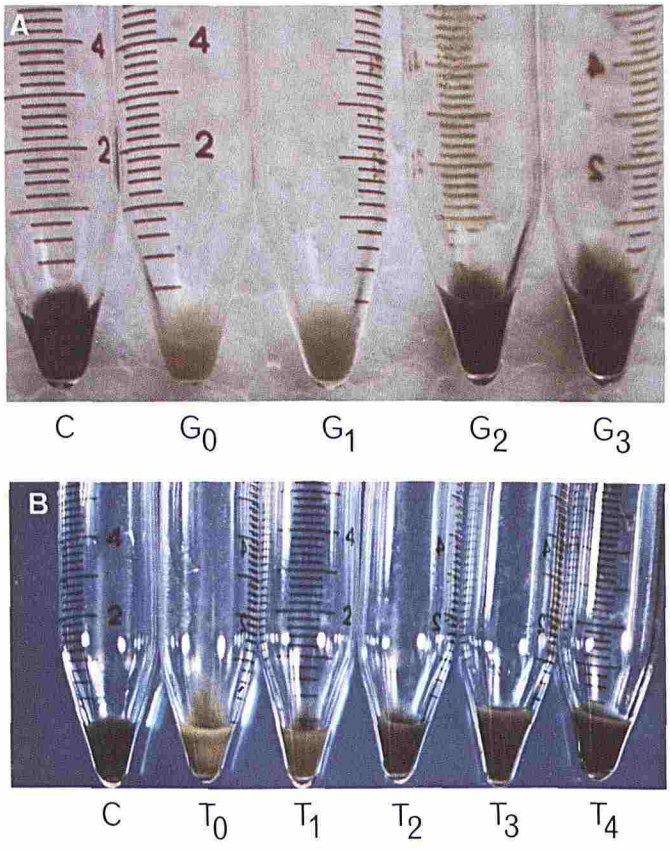

FIG 2. Disappearance and reappearance of melanin pigment in suspensions of pelleted cells following glycosylation inhibitor treatment and the subsequent removal of the inhibitor. B-16 cells were cultured with the glycosylation inhibitor for 11–21 days, inducing complete loss of pigment and the ability to melanize. After the addition of fresh medium without glycosylation inhibitor, the cells were cultured for an additional 3–4 days. *A*, Control, untreated B-16 cells (*C*). Glucosamine (1 mg/ml)-treated B-16 cells on the 11th day of culture (G_0) and subsequent treatment of these cells without glucosamine for 1 (G_1), 2 (G_2), and 3 (G_3) days. *B*, Control, untreated B-16 cells (*C*). Tunicamycin (0.2 ng/ml)-treated B-16 cells on the 21st day of culture (T_0) and subsequent treatment of these cells without tunicamycin for 1 (T_1), 2 (T_2), 3 (T_3), and 4 (T_4) days.

DISCUSSION

The role of carbohydrate moieties in various functioning glycoproteins such as interferon [19], lysosome enzymes [20], fibronectin [21], and procollagen [22] has been of great interest to many investigators. However, the theoretically expected function of carbohydrate moieties of such proteins at the cellular level has not been well clarified in the mammalian cell system. We have recently found that membrane-bound tyrosinase (T_3) within melanogenic compartments is lost by glycosylation inhibition, which results in the loss of melanization with no substantial decrease in the total amount of tyrosinases (T_1, T_2) [6]. One of the key interests in melanogenesis is to clarify the ontogeny of the multiple forms of tyrosinases T_1, T_2, and T_3 and their interrelated dynamics.

Our present recovery experiments are aimed not only at characterizing biochemically the recovery phenomena of melanization after removal of glycosylation inhibition but also at analyzing the initial evolution of tyrosinase isozymes through determination of their varying melanogenic functions. Our present findings have revealed that between 8 and 24 h after removal of glucosamine or tunicamycin, T_3 tyrosinase is already re-formed prior to the substantial recovery of pigmentation of melanoma cells. This also occurs even with the continued level

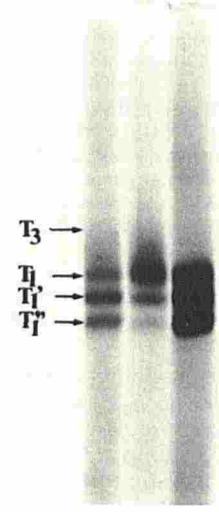

FIG 4. SDS-PAGE of DOC-solubilized LGF from glucosamine-treated B-16 cells in the presence of PMSF and LPT. Tyrosinase activity is stained with dopa reaction after electrophoresis. Photograph of electrophoresis. *Left:* Glc-treated cell. *Center:* Glc-treated cells in the presence of 1 mM PMSF. *Right:* Glc-treated cells in the presence of 0.1 mM LPT.

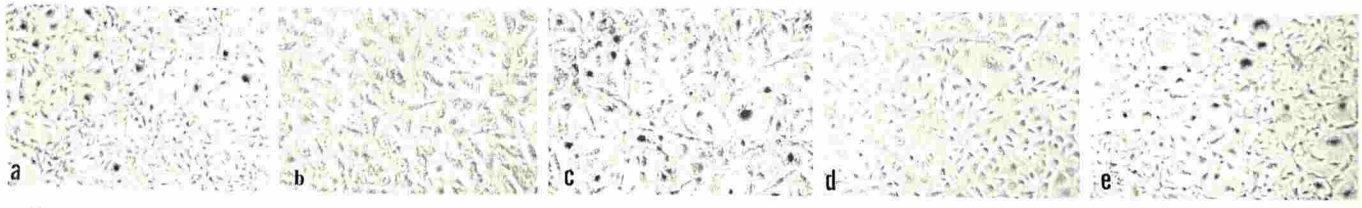

FIG 3. Light microscopic figures of B-16 cells during recovery process of melanin pigment after removal of glycosylation inhibitors. *a*, Control untreated highly melanotic B-16 cells. *b and c*, Glucosamine-induced unpigmented B-16 cells were cultured without glucosamine for an additional 1 or 3 days, respectively. *d and e*, Tunicamycin-induced unpigmented B-16 cells were cultured without tunicamycin for an additional 1 or 3 days, respectively.

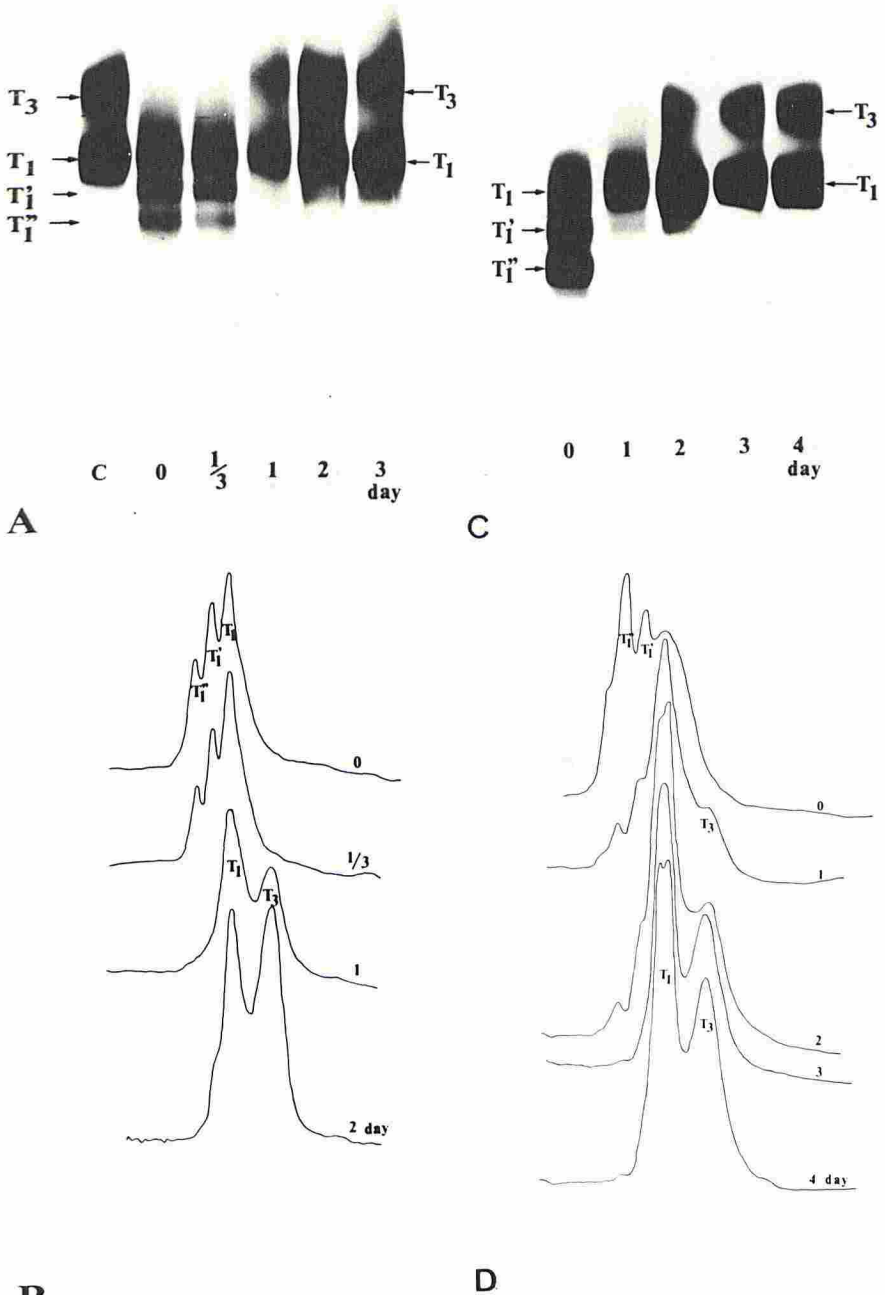

FIG 5. SDS-PAGE of DOC-solubilized LGF from glycosylation inhibitor-treated B-16 cells during the recovery period following interruption of melanogenesis by glucosamine (*A*, *B*) and tunicamycin (*C*, *D*). Tyrosinase activity is made visible with the dopa reaction. *A*, Control, untreated B-16 cells (*c*). Glucosamine-treated B-16 cells 0, ⅓, 1, 2, and 3 days of culture after removal of glucosamine. *B*, Densitometoric scan of SDS-PAGE patterns in *A*. *C*, Tunicamycin-treated B-16 cells 0, 1, 2, 3, and 4 days of culture after removal of tunicamycin. *D*, Densitometric scan of SDS-PAGE patterns in *C*.

of inhibited protein synthesis by cycloheximide, thus suggesting that this recovery process is glycosylation-dependent.

Concerning the biologic role of carbohydrate in glycoproteins, Bernard et al [21] reported that non-glycosylated fibronectin or IgM is more susceptible to pronase or trypsin digestion than are the native counterparts in vitro. Carbohydrates are also considered to protect selectively a certain domain of glycoprotein against proteases and to promote the refolding proteins into a more stable and resistant conformation. However, we found that the addition of protease inhibitors used does not affect the unmelanized process including loss of T_3 tyrosinase and appearance of T_1 heterogeneity, induced by glycosylation inhibitors. These findings do not seem to support the proteolytic conversion from glycosylation-interrupted T_3 tyrosinase to T_1 tyrosinase in our case.

Glycosylation inhibition experiments have indicated that T_1 tyrosinase in the induced unmelanized cells becomes heterogeneous and consists of subclasses of T_1, T_1', T_1'', as characterized by SDS electrophoresis. Further analysis throughout the recovery process has revealed that 8 h after removal of glucosamine, T_3 tyrosinase is still undetectable in melanosome-containing fraction. T_1 heterogeneity also continues to be present, parallel to the remaining unmelanized cells, but is found to decrease 24 h after removal, almost concomitant with T_3 tyrosinase reappearance.

Similarly the reversible induction of altered carbohydrate moieties by glucosamine has also been reported by Pan and Elbein [14]. They demonstrated that at higher concentrations it inhibits the incorporation of [2-^{3}H]mannose into functional viral glycoprotein in influenza virus-infected MDCK cells, resulting in the loss of the $Glc_3Man_9GlcNAc_2$ species and in the appearance of a small $Man_3GlcNAc_2$ species in their lipid-linked oligosaccharides. When the cells were washed free of this inhibitor, they resumed synthesis of $Glc_3Man_9GlcNAc_2$

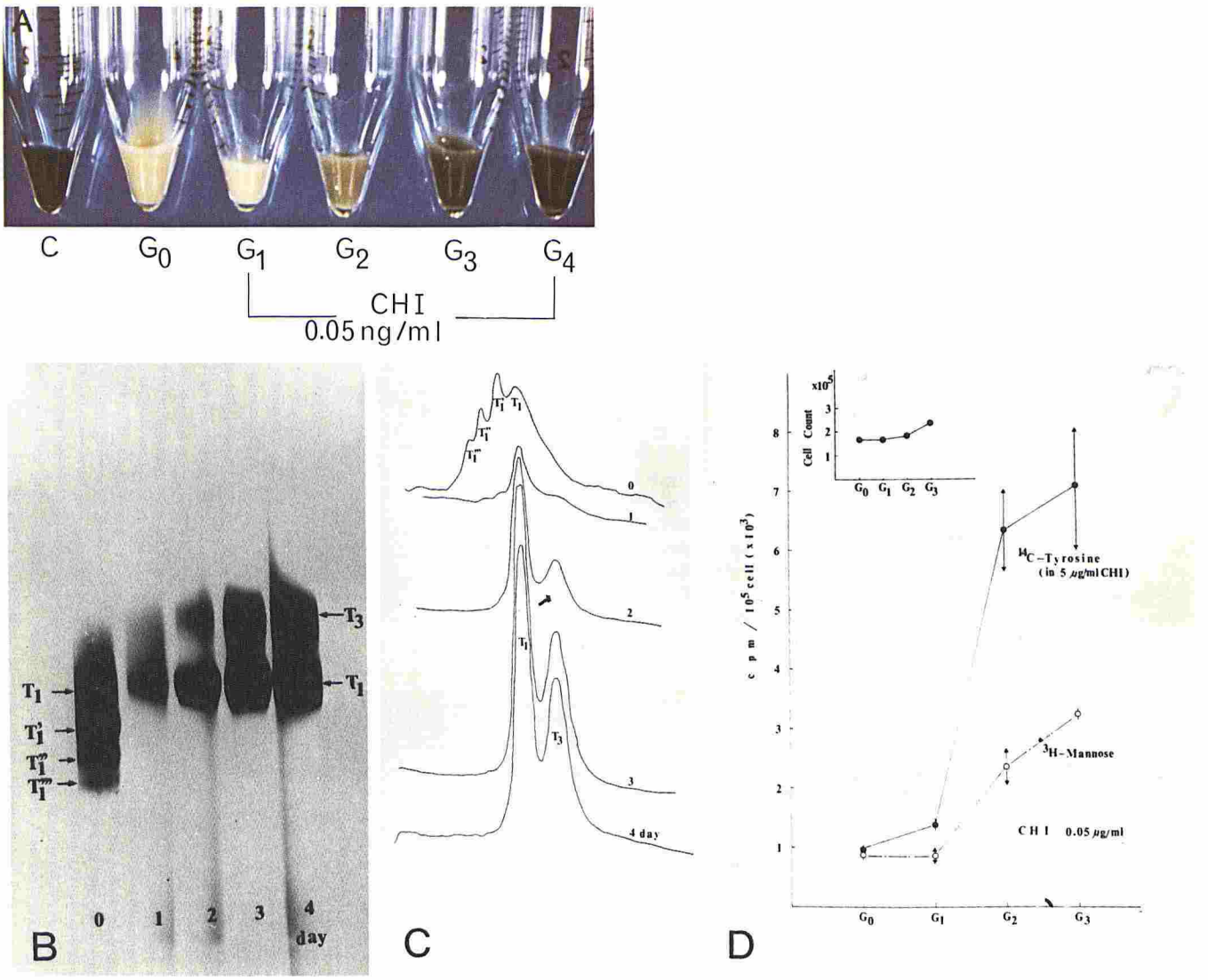

FIG 6. *A*, Reappearance of melanin pigment of the pelleted cells in the recovery process of glycosylation inhibition where protein synthesis is inhibited by cycloheximide up to 45%. The cells were cultured with the glycosylation inhibitors for 11 days, inducing the complete loss of their melanization, and after the addition of fresh medium without glucosamine, were recultured in the presence of cycloheximide (0.05 ng/ml) for a further 3–4 days. Glucosamine (1 mg/ml)-treated B-16 cells on the 11th culture day (G_0) and recultured B-16 cells without glucosamine in the presence of cycloheximide for additional 1 (G_1), 2 (G_2), 3 (G_3), and 4 (G_4) days in comparison to those of untreated B-16 cells (*c*). *B and C*, Parallel SDS gel electrophoresis from DOC-solubilized LGFs of B-16 cells which proceed in the recovery process in the presence of cycloheximide. *B* = Photograph of the electrophoresis; *0, 1, 2, 3,* and *4* represent culture days after removal of glucosamine followed by the addition of cylcoheximide. *C* = Densitometric scan of SDS-PAGE patterns in *B*. *D*, Time course of the recovery of tyrosine and mannose uptakes at the recovery process in the presence of cylcoheximide. Glucosamine (1 mg/ml)-treated B-16 cells on the 11th culture day (G_0) and recultured B-16 cells without glucosamine in the presence of cycloheximide for additional 1 (G_1), 2 (G_2), and 3 (G_3) days.

species and $Man_3GlcNAc_2$ disappeared. Our newly observed T_1' and T_1'' tyrosinases which lost concanavalin A affinitive carbohydrate moiety [23] but still retained dopa reactivity may be the result of the interrupted maturation of T_1 tyrosinase. They are possibly T_3 precursors or pre-T_3 subunits because of the prompt or almost concomitant recovery of T_3 and of T_1 homogeneity. It seems reasonable to assume that these inhibitors visualize the normally nonapparent and transit functional segments of the tyrosinase maturation process which is normally a coordinated physiologic process.

Although why such biphasic and interrelated phenomena of tyrosinases after glycosylation inhibition occur is not yet completely understood, it may indicate that there are two kinds of functional carbohydrate units in tyrosinase molecules: (1) bridge carbohydrates, which bind to the premelanosomal membrane and are a melanogenic functional core, and are found to be selectively interrupted in their synthesis by tunicamycin; and (2) non-bridge carbohydrates whose synthesis is relatively tunicamycin insensitive. Induced heterogeneity of T_1 tyrosinases (T_1', T_1'') may be due to the interrupted synthesis of these bridge carbohydrates. These new T_1 species are electrophoretically identical to but different in molecular weight from the original T_1 tyrosinase. Although it has been considered by some [24,25] that T_3 tyrosinase is merely a membrane-bound form of T_1 tyrosinase, our available evidence suggests that there appear to be additional biologic properties in T_3 tyrosinase which are essential for melanization and for which N-glycosidically linked oligosaccharides play an integral role.

Hearing, Kornor, and Pawelek [5] have recently reported differential functions of multiple forms of tyrosinases in the melanogenesis pathway. Soluble tyrosinase (T_1) contains blocking factor activity which retards the conversion of 5,6-dihy-

droxyindole into melanin, while membrane-bound tyrosinase T_3 found in melanosomes contains activity that accelerates melanin formation from dopachrome. These facts are essentially in agreement with our findings describing a function of the carbohydrate moieties of T_3 tyrosinase necessary for in vivo melanization.

It may be concluded that the initiation of melanization requires the recovery of T_3 tyrosinase within melanosomes which is accompanied by the increased uptake of mannose and re-formation of normally structured melanosomes.† Although we cannot definitely rule out the possibility that re-formation of the T_3 tyrosinase occurs independently of the disappearance of T_1 tyrosinase heterogeneity, evidence obtained here seems to suggest that maturation of tyrosinase occurs from T_1 segments to T_3 tyrosinase primarily by the addition of N-glycosidically linked oligosaccharide moieties which are clearly shown to be functional units of melanization within melanosomes.

† Imokawa G, Mishima Y: Intracellular dynamics of tyrosinases and premelanosomes during recovery of glycosylation inhibition in cultured B-16 melanoma cells. In preparation.

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