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Promotion of Tyrosinase Folding in Cos 7 Cells by Calnexin[1]

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To understand the process of expression of tyrosinase, a key enzyme of melanogenesis, we examined its maturation in the endoplasmic reticulum (ER) by using a heterogeneous expression system. When human tyrosinase cDNA was introduced into COS 7 cells, tyrosinase activity was minimally detected. Immunofluorescence study revealed that tyrosinase was immunolocalized in the nuclear rim, the reticular network, and the punctuated structures. Because a cytoplasmic tail of tyrosinase-gene family protein functions as a lysosomal targeting signal in non-melanocytic cells, and immature and/or misfolded molecules are selectively retained in the ER, the observed localization suggested the inefficient maturation in the COS 7 cells. We thus examined if supplementation of calnexin, a membrane-bound chaperone with affinity for oligosaccharide-processing intermediates containing monoglucose, could improve the process. As expected, the activity was enhanced ~2-fold by co-transfection of cDNA encoding calnexin. In contrast, co-transfection of the cytosolic tail-free calnexin, which inhibits calnexin function by allowing premature egress of its ligands from the ER, suppressed expression of this enhanced tyrosinase activity. When α-glucosidase activity, which is required for calnexin function, was inhibited by castanospermine (CST) treatment, expression of tyrosinase activity was completely abolished. To confirm the direct involvement of calnexin in tyrosinase maturation, the interaction of calnexin with tyrosinase was examined. Immunoprecipitation of calnexin from extracts of [^{35}S]methionine labeled cells with anti-calnexin antibody revealed that the association is highest immediately after the pulse and that nascent tyrosinase is gradually dissociated upon chase. The association was completely inhibited when CST was included in the medium. Hence, we suggest that the proper folding of tyrosinase is largely dependent on its direct interaction with calnexin for the determined duration in the ER.

Key words: calnexin, melanocyte, melanogenesis, melanin, tyrosinase.

Melanosomes are highly specialized organelles where melanin is synthesized and deposited on their internal matrix (*1-3*). Several proteins are closely associated with the melanosomal membrane or contained within melanosomes: tyrosinase (*4*), tyrosinase-related protein (TRP)-1/gp75 (*5*), HMSA-5 (*6*), TRP-2 (*7*), silver locus/gp100 (*8, 9*), pink eye protein (*10, 11*), HMSA-1/2 (*12, 13*), and MART-1 (*14*). With the exception of MART-1, all proteins are involved in regulation of melanogenesis (*15*).

We have previously cloned p90, Ca^{2+}-binding phosphoprotein, from human melanoma cDNA library using a polyclonal antibody against human melanosomes (*16*). Immuno-electron microscopy and histochemistry revealed p90 to be localized primarily in the endoplasmic reticulum (ER) and associated with the melanosomal membrane, indicating its involvement in melanogenesis (*17, 18*). Sequence analysis of p90 cDNA revealed 100% identity with human calnexin (*19*). Calnexin, a type I membrane protein, functions as a molecular chaperone which associates specifically with incompletely folded or unassembled glycoproteins and facilitates their acquisition of correct three-dimensional protein structure (*20*).

Recently several groups have reported that calnexin possesses affinity for $Glu_1Man_{9\text{-}6}GluNAc_2$, which is a substrate of glucosidase I and II, and that it can be regenerated from deglucosylated oligosaccharides by the action of UDP-glucose, namely, glycoprotein glucosyltransferase (*21-23*). The dissociation and re-association cycles are essential for the chaperone function of calnexin (*22*). Tyrosinase is the key enzyme in melanin synthesis, having a dual catalytic function in the initial step of melanin synthesis, *i.e.*, hydroxylation of tyrosine to dopa (tyrosine hydroxylase activity), and subsequent oxidation of dopa to dopa-quinone (dopa oxidase activity), and also an indole-

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conversion function in the late step of melanin synthesis (*24-26*). Tyrosinase is *N*-glycosylated in the ER and modified further in the Golgi apparatus. Because glycosylation inhibitors induce loss of pigmentation in melanocytic cells, glycosylation is believed to be essential for tyrosinase to be translocated into melanosomes and to induce melanogenesis within the melanosomal compartment (*27*). However, the folding process, which is an essential step in acquisition of enzyme activity, is unknown. In this study, we employed a heterologous expression system using COS7 cells and examined the role of calnexin in tyrosinase maturation.

MATERIALS AND METHODS

Cell Line—COS7 cells (African green monkey kidney cells, CRL 1650, American Type Culture Collection) were cultured in Dulbecco's modified Eagle medium (D-MEM) containing 10% fetal calf serum and penicillin-streptomycin (100 μg/ml).

Antibodies—Anti-calnexin monoclonal and polyclonal antibodies were purchased from StressGen (Victoria, Canada). Rabbit polyclonal anti-tyrosinase antibody (αPEP-7) was a gift from Dr. Vincent J. Hearing, National Institute of Health, Bethesda, USA (*28*). Mouse monoclonal anti-human tyrosinase (NCL-TYROS) was purchased from Novocastra Laboratories (Newcastle, UK). Antibodies against a luminal domain and rat lgp96 were raised in rabbit. Alexa 595-labeled goat anti-mouse IgG [F(ab)$_2$] and Alexa 488-labeled goat anti-rabbit IgG [F(ab)$_2$] were purchased from Molecular Probe (Eugene, OR).

Expression Vector—Wild-type (wt) calnexin and cytoplasmic tail (CYT; 89 amino acids)-deleted calnexin cloned into the expression vector AP^{7}M8 were the gift of Dr. Michael Brenner, Harvard Medical School, Boston, USA (*29*). Human tyrosinase expression vector was constructed as follows: the plasmid pBBTY-1 encoding the full length human tyrosinase cDNA was excised by *Eco*RI restriction enzyme, and the *Eco*RI-digested cDNA fragment was subcloned into *Eco*RI site of pcDNA3 expression vector (Invitrogen, San Diego, CA) containing the CMV promoter. Transient cDNA expression in COS7 cells was performed following transfection by the calcium phosphate method (*30*). Briefly, cells grown to 50-60% confluence in 100-mm dishes were incubated with expression vector in a mixture containing $CaCl_2$ and BBS buffer (50 mM BES; *N,N*-bis-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.9) in an atmosphere of 3.0% CO_2 for 24 h. They were then washed three times with phosphate-buffered saline (PBS) (pH 6.9), incubated in fresh D-MEM containing 10% fetal calf serum at 5.0% CO_2, and harvested.

Metabolic Labeling and Immunoprecipitation—Metabolic labeling and immunoprecipitation followed our previous method with some modification (*6*). Briefly, cells were cultured in methionine-depleted D-MEM, then labeled at 37°C with [^{35}S]methionine (Life Science Amersham, Ontario, Canada). For pulse-chase experiments, cells were labeled for 15 min, then chases were performed for specific periods in D-MEM supplemented with 1 mM L-methionine. Harvested cells were washed twice with ice-cold PBS and solubilized in cold RIPA buffer (150 mM NaCl, 0.5% deoxycholic acid, 1% NP40, 0.1% SDS, 50 mM Tris-HCl, pH 7.5), containing protease inhibitors (4-amidinophenyl methanesulfonyl fluoride, pepstatin, and leupeptin) for 1 h on ice. For precipitation, the clarified supernatant was incubated with rabbit serum-agarose (Sigma Immuno Chemical, St. Louis, MO, USA) and Protein A-Sepharose for 1 h at 4°C. Protein A-Sepharose was collected by centrifugation at 4°C, and samples of supernatant were incubated with anti-tyrosinase (αPEP-7) or anti-calnexin antibody at 4°C overnight with end-over-end rotation.

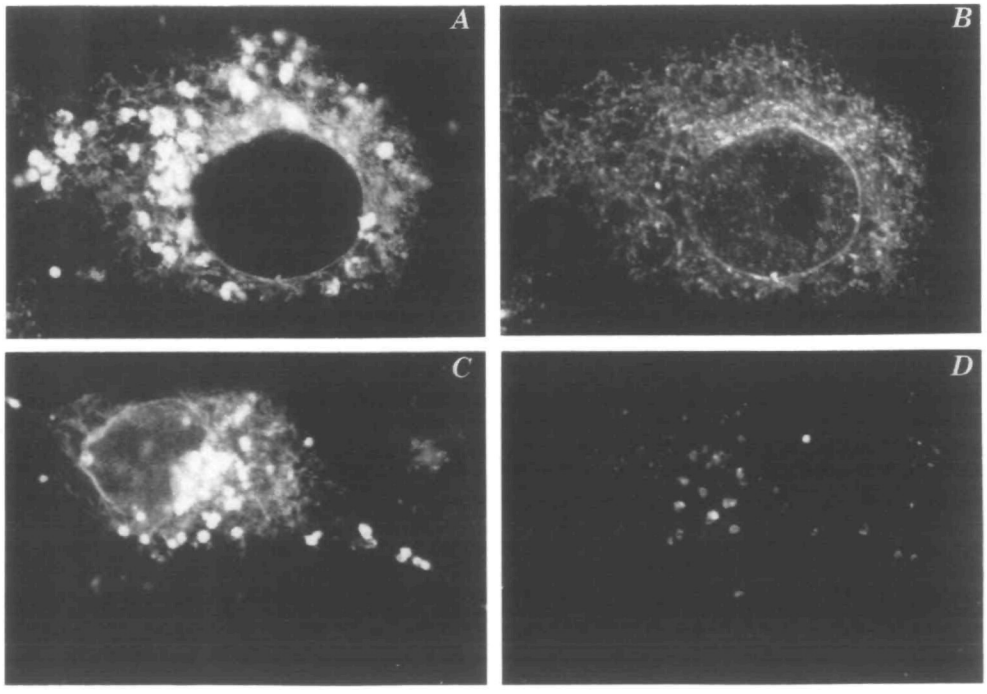

Fig. 1. **Localization of tyrosinase expressed in Cos7 cells.** Cos7 cells expressing tyrosinase was immuno-stained with anti-tyrosinase antibody and Alexa 595 labeled goat-anti-mouse IgG[F(ab)$_2$] (Panels A and C). The cell were sequentially stained with anti-calnexin polyclonal antibody (Panel B), or anti lgp 96 polyclonal antibody (Panel D) and Alexa 488 labeled goat-anti-rabbit IgG [F(ab)$_2$]. The same views (Panel A and C or Panel D) under Laser Scanning Confocal Microscopy with a filter set for Texas Red (Panel A and C) and FITC (Panel B and D) were shown. ×2,400.

Protein A-Sepharose was added to the samples, which were then incubated at 4°C with end-over-end rotation for 1 h. Protein A-Sepharose was pelleted as immune complex and washed with 1% NP40 in washing buffer (250 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5). The final pellet was resuspended in sample buffer, heated at 95°C for 5 min, and centrifuged to remove Protein A-Sepharose. Samples were reheated at 95°C for 5 min prior to analysis by SDS-PAGE (10%) and fluorography. Kodak X-omat AR X-ray film (Eastman Kodak, Rochester, NY, USA) was used to visualize the labeled protein.

Tyrosinase (Dopa Oxidase) Assay—Tyrosinase was assayed as described previously (*31*). Cell lysate was prepared with 100 μl of phosphate buffer (0.1 M, pH 6.8) containing 1% (v/v) Triton X 100. Supernatant was collected by centrifugation, and 10 μg of protein was incubated with phosphate buffer with 0.15% of dopa (w/v). Samples were incubated at 37°C for 30 min and optical density was measured spectrophotometrically at 475 nm.

Dopa Staining, Indirect Immunofluorescence, and Confocal Microscopy—Dopa staining was done according to the method described previously with some modification (*32*). Cells were fixed with acetone/methanol fixation solution (ratio, 1:1) for 30 s, washed with PBS, then incubated with dopa solution [0.15% (w/v) L-dopa in 0.1 M phosphate buffer, pH 6.8], at 37°C for 20 min. After washing with PBS, cells were examined under a light microscope (Model TMS-F, Nikon, Tokyo) and photographed. To further examine the biodistribution of tyrosinase, the transfected cells were immunostained with anti-tyrosinase monoclonal antibody (NCL-TYROS) and observed with an Olympus Laser Scanning Confocal Imaging System (Fluoview, Olympus, Tokyo) after counterstaining of nuclei with propidium iodide. The tissue preparation and indirect immunofluorescence followed our previous report (*16*).

Melanin Content—Melanin within cells including transfected cells (at least 1×10^6 cells) was solubilized in 1 ml of 0.2 N NaOH, and measured spectrophotometrically at an optical density of 475 nm, as described by Lee *et al.* (*33*).

Statistical Analysis—The statistical significance of differences between mean values was determined using Student's *t*-test.

RESULTS

Expression of tyrosinase activity by COS7 cells transfected with human tyrosinase cDNA.

We initially examined the feasibility of studying tyrosinase function in non-pigmental cells such as COS7 cells. To this end, we transfected COS7 cells with human tyrosinase cDNA and examined the expression of tyrosinase activity by *in situ* staining using the dopa reaction, tyrosinase protein expression by indirect immunofluorescence confocal microscopy, and *in vitro* melanin formation. At 48 h post-transfection, tyrosinase protein was expressed in COS7 cells (Fig. 1, A and C). The expressed enzyme, as measured by dopa reaction, showed the ability to synthesize melanin by light microscopy, respectively (Fig. 2A). The immunoreactivity (Fig. 1) indicative of tyrosinase protein expression was seen under confocal microscopy as granular and scattered over the entire perikaryon, with a similar distribution to that of lysosomes, and membranous around the perinuclear rim and reticular with a similar distribution to that of the ER (*16*). Most of the former punctuated structure was co-stained with antibody against lgp96, one of the major lysosomal glycoproteins. The reticular structure was also revealed by immunostaining with anti-calnexin antibody (Fig. 1B). The intensity of dopa staining was not significantly increased 78 h post-transfection (compare Fig. 2A with Fig. 2C). When the activity was quantitated by the *in vitro* assay using the cell lysates, COS7 cells transfected with human tyrosinase cDNA showed a 5.8-fold higher tyrosinase activity (0.187 ± 0.031 ΔE475/20 min/10 mg protein) than COS7 cells transfected with control plasmid + pcDNA3 (0.032 ± 0.005 ΔE475/

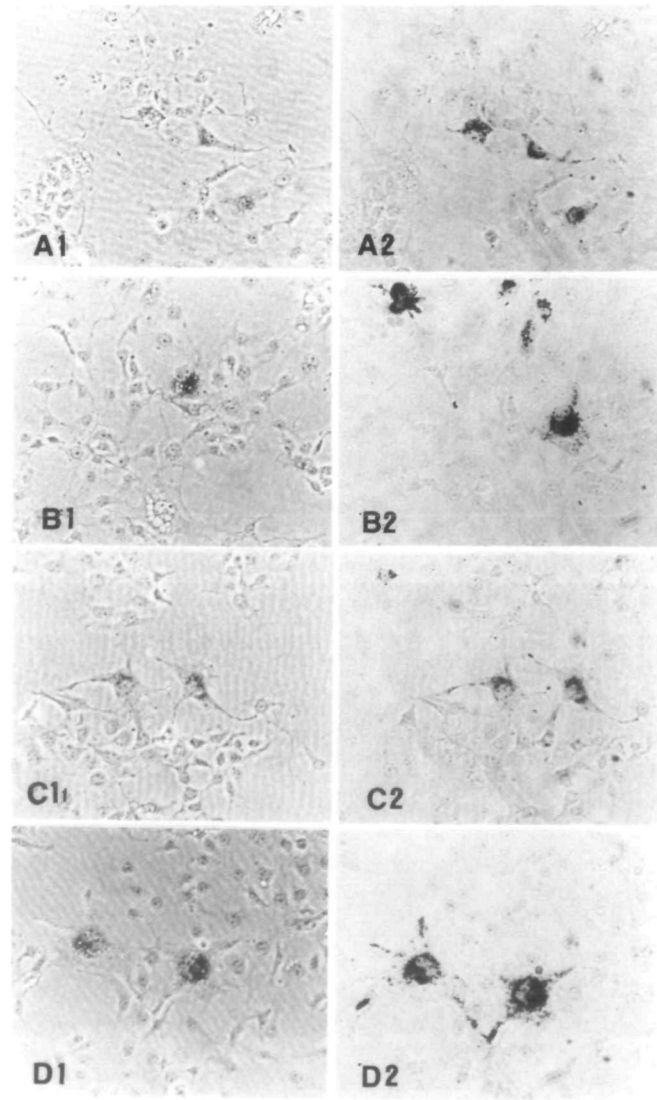

Fig. 2. **Expression of tyrosinase (dopa oxidase antibody) in COS7 cells.** Transfected cells were incubated with dopa to visualize tyrosinase activity *in situ* as described under "MATERIALS AND METHODS." Micrographs were taken without (1: left) or with (2: right) the slit which reduces the enhancement of light contrast. (Magnification: ×200). (A) COS7 cells transfected with tyrosinase cDNA and pcDNA3, 48 h after transfection. (B) COS7 cells transfected with tyrosinase and calnexin cDNAs, 48 h after transfection. (C) COS7 cells transfected with tyrosinase cDNA and pcDNA3, 72 h after transfection. (D) COS7 cells transfected with tyrosinase and calnexin cDNAs, 72 h after transfection.

20 min/10 mg protein) (Fig. 3A). Lysates of COS7 cells transfected with human tyrosinase cDNA contained 1.56 ± 0.15 pg of melanin/10^6 cells, while the COS7 cells transfected with pcDNA3 contained virtually no melanin (0.01 ± 0.00 pg/10^6 cells) (Fig. 3B).

A portion of the expressed tyrosinase was apparently targetted to the lysosome-like structures, to which a cytoplasmic tail of tyrosinase-gene family protein is reportedly directed. However, a significant population of the expressed tyrosinase was observed in the ER, suggesting that the maturation process may be inefficient in this system. We reasoned that this may be due to insufficiency of certain molecular chaperones in COS 7 cells. We thus examined if supplementation of calnexin would improve the tyrosinase maturation. When calnexin cDNA was co-transfected with tyrosinase cDNA into COS7 cells, formation of brown-black granules increased over that of COS7 cells transfected with tyrosinase cDNA and pcDNA3 (compare Fig. 2A with Fig. 2B). The lysates prepared from co-transfected cells showed a 2.4-fold enhancement of tyrosinase activity (0.451 ± 0.118 ⊿E475/20 min/10 mg protein). Melanin contents in the cells were also increased by 1.8-fold (2.8 ± 0.32 pg/10^6 cells). Interestingly, expression of tyrosinase activity continued to rise up to 96 h post-transfection by calnexin expression (Fig. 3C). At 72 h of post-transfection, COS7 cell co-transfected with tyrosinase and calnexin cDNAs were also stained more intensely by the dopa reaction than those cells 48 h after transfection

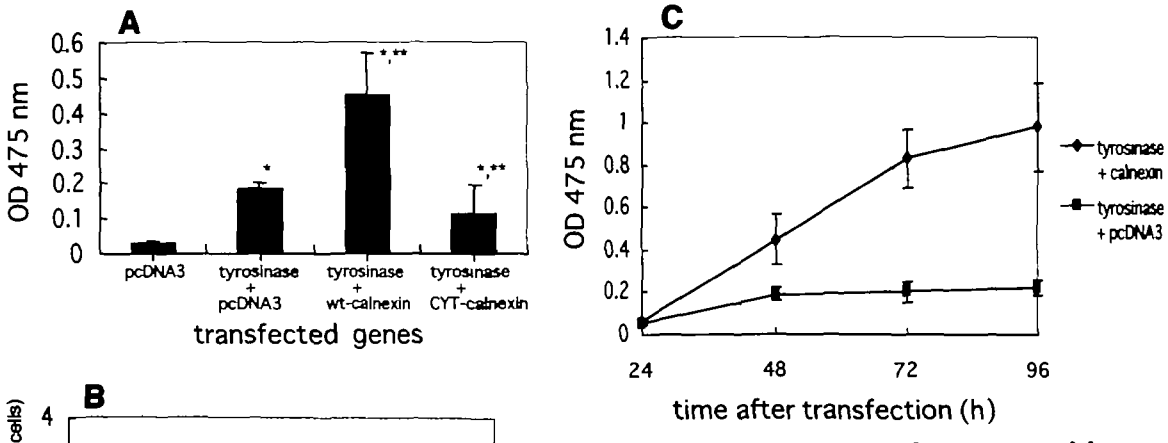

Fig. 3. **Tyrosinase activity and melanin content of the transfected COS7 cell lysates.** COS7 cells were transfected with pcDNA3 alone, tyrosinase cDNA and pcDNA3, tyrosinase and wild-type (wt) calnexin cDNAs, or tyrosinase and cytoplasmic tail (CYT)-deleted calnexin. Tyrosinase activity (dopa activity) and melanin content were measured as described under "MATERIALS AND METHODS." (A) Tyrosinase activity of transfected COS7 cells 48 h after transfection. $*p<0.05$: tyrosinase activity of COS7 cells transfected with pcDNA3 alone. $**p<0.05$: tyrosinase activity of COS7 cells transfected with tyrosinase cDNA and pcDNA3. (B) Melanin content of the transfected COS7 cells at 48 h post-transfection. (C) Continuous measurement of tyrosinase activity of the transfected COS7 cells with tyrosinase and calnexin cDNAs (◆) or tyrosinase cDNA and pcDNA3 (■) up to 96 h. Data are expressed as means ± SD.

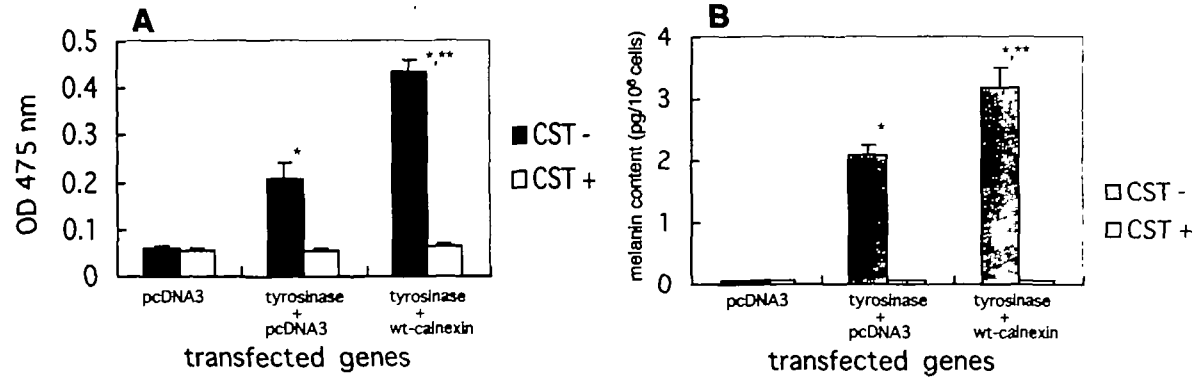

Fig. 4. **Castanospermine treatment abolished expression of tyrosinase activity.** COS7 cells were transfected with pcDNA3 alone, tyrosinase cDNA and pcDNA3, or tyrosinase and calnexin. Transfected cells were incubated in the absence or presence of 200 μM castanospermine (CST). Tyrosinase activity (dopa activity) and melanin content were measured as described under "MATERIALS AND METHODS." (A) Tyrosinase activity of transfected COS7 cells 48 h after transfection. $*p<0.05$: tyrosinase activity of COS7 cells transfected with pcDNA3 alone. $**p<0.05$: tyrosinase activity of COS7 cells transfected with tyrosinase cDNA and pcDNA3. (B) Melanin content of transfected COS7 cells 48 h after transfection. Data are expressed as means ± SD.

(compare Fig. 2B with Fig. 2D). This is in contrast to the cells without calnexin co-expression, where the activity reached plateau 48 h after transfection (Fig. 3C).

It was previously reported that the cytosolic tail of calnexin is needed for the ER localization, and the lack of this domain causes plasmalemmal localization of calnexin in COS7 cells (*29*). If maturation of tyrosinase requires interaction with calnexin for the determined duration in the ER, then over-expression of cytoplasmic tail (CYT)-deleted calnexin should competitively inhibit tyrosinase folding. When co-transfected with CYT-deleted calnexin cDNA and tyrosinase cDNA, COS7 cells showed 55% lower tyrosinase activity (0.085 ± 0.014: ΔE475/20 min/10 mg protein) than those cells transfected with tyrosinase and wild type (wt) calnexin cDNAs (0.187 ± 0.03 ΔE475/20 min/10 mg protein) (Fig. 3A).

Effect of Modification of Glycosylation on Tyrosinase Activity—Calnexin shows affinity for monoglucosylated asparagine-like oligosaccharides ($Glu_1Man_{9\text{-}8}GlucNAc_2$), which are formed by removal of the first and second glucoses from the original triglucosylated core oligosaccharide ($Glu_3Man_9GlucNAc_2$) by the sequential action of glucosidase I and II (*22, 23, 35*). The monoglucosylated oligosaccharides are also formed by post-translational glucosylation of non-glucosylated oligosaccharides. If chaperone function of calnexin is critical for proper folding of tyrosinase, inhibition of glucosidases should abolish the expression of tyrosinase activity. Hence, we studied the effect of an inhibitor, castanospermine (CST), on expression of tyrosinase activity in COS7 cells. COS7 cells transfected with pcDNA3 alone, with tyrosinase cDNA and pcDNA3, or with tyrosinase and wt-calnexin cDNAs were incubated with or without 200 μM CST after transfection, and then tyrosinase activity was measured 48 h post-transfection (Fig. 4A). When COS7 cells transfected with tyrosinase cDNA and pcDNA3 or with tyrosinase and calnexin cDNAs were treated with CST, the expression of tyrosinase activity was completely abolished (from 0.209 ± 0.032 to 0.053 ± 0.007, or from 0.434 ± 0.028 to 0.062 ± 0.008 ΔE475/20 min/10 mg protein, respectively). As a result, melanin was not detected in any of the CST-treated cells, while they were capable of synthesizing melanin without CST treatment (2.1 ± 0.27 and 3.2 ± 0.33 pg/10^6 cells, respectively) (Fig. 4B). On the other hands, treatment with 200 μM CST did not influence the cell growth (data not shown).

Pulse-Chase Experiment—To demonstrate the physical interaction of nascent tyrosinase with calnexin, we pulse-labeled the transfected cells and analyzed it by using immunoprecipitation (Fig. 5A). The endogenous calnexin was detected as a 90-kDa protein by anti-calnexin antibody on SDS-PAGE gel (Fig. 5A, lane a, c), whereas no tyrosinase bands were detected by anti-tyrosinase antibody in COS7 cells transfected with pcDNA3 (Fig. 5A, lane a, t). When COS7 cells were transfected with tyrosinase cDNA and pcDNA3, a triplet (one major band and two minor bands) of tyrosinase bands was detected at 69-75 kDa by anti-tyrosinase antibody (Fig. 5A, lane b, t). A set of triplet was co-immunoprecipitated by anti-calnexin antibody (Fig. 5A, c of lane b). When COS7 cells were transfected with wt-calnexin cDNA and pcDNA3, calnexin (Fig. 5A, lane c, c) but no tyrosinase (Fig. 5A, lane c, t) was precipitated. In COS7 cells transfected with tyrosinase and wt-calnexin

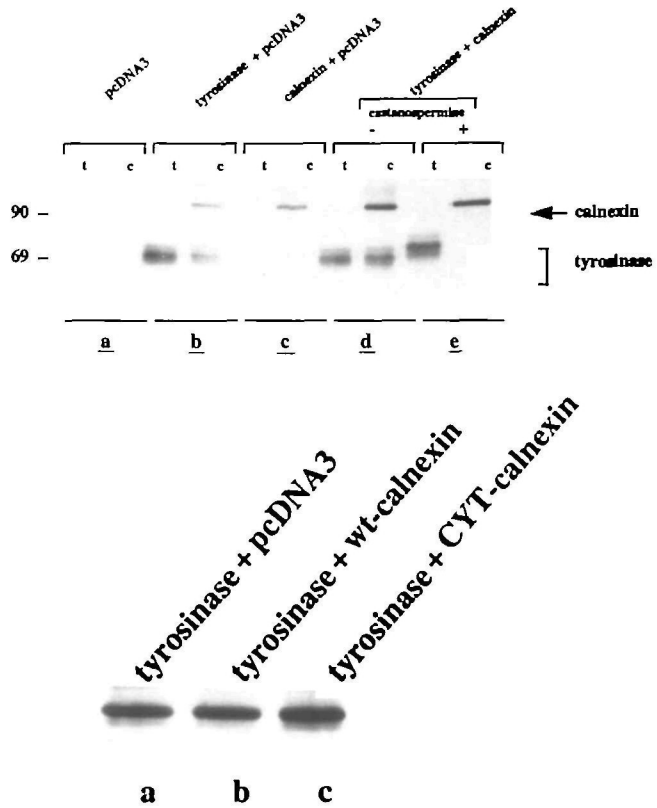

Fig. 5. **SDS-PAGE analysis of the immunoprecipitates.** (A) The lysates from cells were immunoprecipitated with anti-tyrosinase antibody (t) or anti-calnexin antibody (c), then immune complexes were analyzed by SDS-PAGE using 10% gel. COS7 cells were transfected with pcDNA3 alone (a), tyrosinase cDNA and pcDNA3 (b), wt-calnexin cDNA and pcDNA3 (c), or tyrosinase and calnexin cDNAs (d and e). COS7 cells transfected with tyrosinase and calnexin cDNAs were incubated at the presence of 200 μM castanospermine (CST) (d) or in the absence of CST (e) throughout the transfection. (B) The lysates from cells were immunoprecipitated with anti-tyrosinase antibody. COS7 cells were transfected with tyrosinase cDNA and pcDNA3 (a), tyrosinase and wt-calnexin cDNAs (b), or tyrosinase and CYT-deleted calnexin cDNAs (c).

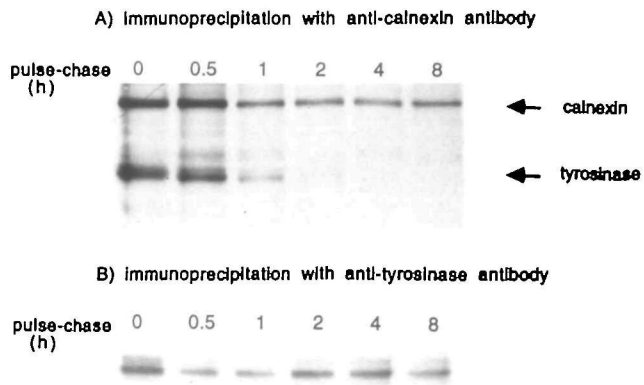

Fig. 6. **Transient interaction of tyrosinase with calnexin.** COS7 cells were transfected with tyrosinase and calnexin cDNAs. Transfectants were labeled and chased (up to 8 h). Calnexin or tyrosinase were recovered by use of anti-calnexin (A) or anti-tyrosinase (B) antibody and analyzed by SDS-PAGE using 10% gel.

cDNAs, anti-calnexin antibody precipitated calnexin and enhanced the amount of a triplet which co-migrated with tyrosinase precipitated by anti-tyrosinase antibody (Fig. 5A, lane d, c). When COS7 cells transfected with tyrosinase and wt-calnexin cDNAs were incubated with CST, the binding of tyrosinase to calnexin was completely inhibited (Fig. 5A, lane e, c) despite high tyrosinase expression (Fig. 5A, lane e, t). Tyrosinase synthesized in the presence of CST (Fig. 5A, lane d, t) migrated more slowly than that in untreated cells (Fig. 5A, lane e, t), consistent with the effect of α-glucosidase inhibition. To confirm that the expression of tyrosinase is not affected by co-transfected cDNA, tyrosinase was immunoprecipitated with anti-tyrosinase antibody in COS7 cells transfected with tyrosinase cDNA and either pcDNA3, wt-calnexin cDNA, or CYT-deleted calnexin cDNA (Fig. 5B). Expression levels of each transfectant were comparable.

We finally performed a pulse-chase experiment to determine kinetics of the interaction (Fig. 6). COS7 cells were labeled for 15 min with [^{35}S]methionin, then chased for 0, 0.5, 1, 2, 4, and 8 h. Recovery of tyrosinase in the calnexin complex was the highest immediately after the chase and gradually decreased with chase time. At 8 h, no tyrosinase was found in association with calnexin.

DISCUSSION

Tyrosinase is a critical and rate-limiting enzyme in melanogenesis (*1-3*). To elucidate its maturation process, which is largely unknown, COS7 cells, which do not express any tyrosinase, were transfected with tyrosinase cDNA in this study. Unlike in melanocytic cells, where tyrosinase is normally transported to melanosomes (*34*), tyrosinase is sorted to lysosomes and expresses enough enzymatic activity to produce pigmentation in non-pigmented cells including COS7 cells (*35*). In the present study, we focused on the role of calnexin during tyrosinase maturation, because it was previously shown that inhibition of *N*-glycosylation abolishes melanin synthesis (*27*). We report here that the chaperone function of calnexin is indispensable for the expression of tyrosinase activity in COS7 cells. This conclusion is based on three lines of evidence: (a) the amount and duration of expression of tyrosinase activity in COS7 cells were enhanced by co-transfection of calnexin cDNA, (b) the promotive effect was not observed when the mutant calnexin lacking a cytosolic tail was co-expressed, (c) α-glucosidase activity was required for the expression of tyrosinase activity. Although calnexin is involved in the folding and assembly of some proteins including major histocompatibility complex (MHC) class I, influenza hemagglutinin, nicotinic acetylcholine receptor, and vesicular stomatitis virus (VSV) glycoprotein (G protein) (*35, 37-41*), the present finding is surprising because calnexin is not essential for expression of most glycoproteins. For example mutant cells lacking glucosidase I or II are able to synthesize glycoproteins so that the cells grow normally in culture (*42*). It was suggested that BiP may substitute for calnexin/calreticulin in the assembly of MHC class I molecules in a glucosidase II-deficient cell line (*43*). Chen *et al.* has also found that CST merely induced a temporary folding defect during co-translational disulfide bond formation in the folding of HA (*44*). Indeed, CST treatment did not inhibit the growth of COS7 cells. Although it is generally thought that chaperone functions in the ER are rather redundant and we have obtained no clear evidence that calnexin is uniquely important, we think that BiP or other heat-shock proteins may not substitute for calnexin in the current system, because CST treatment is known to elevate levels of heat-shock proteins (*44, 45*). This study, however, has for first time demonstrated the importance of calnexin function in melanogenesis.

We used CYT-free calnexin to examine the role of endogenous calnexin on tyrosinase maturation. Without the cytoplasmic tail, the mutant molecules are transported by default to the cell surface (*29*). However, as the luminal domain is sufficient for binding of the nascent molecules *via* *N*-linked oligosaccharides, overexpression of the CYT-free calnexin should disturb the normal chaperone cycles of the endogenous calnexin by releasing the premature calnexin substrates from the ER to the later secretory pathway. In the COS7 cells expressing tyrosinase, co-expression of CYT-free calnexin reduced the activity to nearly a half (Fig. 3A). Since overexpression of intact calnexin increased tyrosinase activity, this reduction in the tyrosinase activity indicates that correct maturation process of tyrosinase should involve the post-translational cyclic interaction of calnexin. Interestingly, the inhibition was less prominent than that caused by treatment with CST, which nearly abolished the activity (Fig. 4A). The difference may reflect the complete loss of the interaction by the CST treatment, whereas overexpression of CYT-free calnexin alters only the kinetics of the interaction. It is also conceivable that calreticulin, a luminal homologue of calnexin, may also be involved in the process, so that CYT-free calnexin did not abolish the tyrosinase maturation, while CST did. However, this possibility appears unlikely, because little tyrosinase was coimmunoprecipitated by the calreticulin antibody (data not shown).

Melanosomes are considered to be specialized lysosomes (*1-3*). Some of the earliest evidence for the concept was provided by the studies of such genetic diseases as Chediak-Higashi syndrome (*46, 47*), Hermansky-Pudlak syndrome (*48*), and infantile sialic acid storage disease (*49, 50*). Furthermore, by analyzing the intracellular transport of genetically engineered chimeric molecules of CD8 and gp75, Vijiyasaradhi and co-workers suggested that targetting signals for melanosomes and lysosomes are exchangeable and functionally equivalent (*51*). Thus, it is reasonable to assume that biogenesis of exogenous tyrosinase transfected into non-melanotic cells should be comparable to that in melanocytic cells, and we suggest that this experimental system should be useful for studying the molecular mechanism of melanogenesis.

REFERENCES

1. Jimbow, K., Fitzpatrick, T.B., and Wick, M. (1991) Biochemistry and physiology of melanin pigmentation in *Physiology and Molecular Biology of the Skin*, Vol. II (Goldsmith, L.A. eds.) pp. 873-909, Oxford University Press, New York
2. Prota, G. (1992) *Melanins and Melanogenesis*, pp. 1-290, Academic Press, New York
3. Jimbow, K., Lee, S.K., King, M.G., Hara, H., Chen, H., Dakour, J., and Marusyk, H. (1993) Melanin pigments and melanosomal proteins as differentiation markers unique to normal and neoplastic melanocyte. *J. Invest. Dermatol.* **100**, 259-268
4. Kikuchi, H., Miura, H., Yamamoto, H., Takeuchi, T., Dei, T., and

Watanabe, M. (1989) Characteristic sequences in the upstream region of the human tyrosinase gene. *Biochim. Biophys. Acta* **1009**, 283-286
5. Vijayasaradhi, S., Bouchard, B., and Houghton, A.N. (1990) The melanoma antigen gp75 is the human homologue of the mouse b (brown) locus gene product. *J. Exp. Med.* **171**, 1375-1380
6. Der, J.E., Dixon, W.T., Jimbow, K., and Horikoshi, T. (1993) A murine monoclonal antibody, MoAb HMSA-5, against a melanosomal component highly expressed in early stages, and common to normal and neoplastic melanocytes. *Br. J. Cancer* **67**, 47-57
7. Jackson, I.J., Chambers, D.M., Tsukamoto, K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Hearing, V.J. (1992) Second tyrosinase-related protein, TRP-2, map to and is mutated at the mouse slaty locus. *EMBO J.* **11**, 527-535
8. Kwon, B.S., Chintamaneni, C., Kozak, C.A., Copeland, N.G., Gilbert, D.J., Jenkins, N., Barton, D., Francke, U., Kobayashi, Y., and Kim, K.K. (1991) A melanocyte specific gene, Pmel 17, maps near the silver coat color locus on mouse chromosome 10 and is in a synthetic region on human chromosome 12. *Proc. Natl. Acad. Sci. USA* **88**, 9228-9232
9. Adema, G.J., de Boer, A.J., Vogel, A.M., Loenen, W.A.M., and Figdor, C.G. (1994) Molecular characterization of the malanocyte lineage-specific antigen gp100. *J. Biol. Chem.* **269**, 20126-20133
10. Gardner, J.M., Nakatsu, Y., Gondo, Y., Lee, S., Lyon, M.F., King, R.A., and Brilliant, M.H. (1992) The mouse pink-eyed dilution gene: association human Preder-Willi and Angelman syndromes. *Science* **257**, 1121-1124
11. Rinchik, E.M., Bultman, S.J., Hrsthemke, B., Lee, S.T., Strunk, K.M., Spritz, R.A., Avidano, K.M., Jong, M.T.C., and Nicholls, R.D. (1993) A gene for the mouse pink-eyed dilution locus and for human type II oculocutaneous albinism. *Nature* **36**, 72-76
12. Akutsu, Y. and Jimbow, K. (1986) Development and characterization of a mouse monoclonal antibody, MoAb HMSA-1, against melanosomal fraction of human malignant melanoma. *Cancer Res.* **46**, 2904-2911
13. Maeda, K. and Jimbow, K. (1987) Development of MoAb HMSA-2 for melanosomes of human melanoma and its application to immunohistopathologic diagnosis of neoplastic melanocytes. *Cancer* **59**, 415-423
14. Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P.F., Rivoltini, L., Yannelli, J.R., Appella, E., and Rosenberg, S.A. (1988) Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp. Med.* **180**, 1301-1309
15. Hearing, V.J. (1996) Melanogenic proteins as specific melanoma antigen in *Melanogenesis and Malignant Melanoma: Biochemistry, Cell Biology, Molecular Biology, Pathophysiology, Diagnosis and Treatment* (Hori, Y., Hearing, V.J., and Nakayama, J., eds.) pp. 135-148, Elsevier, Amsterdam
16. Dakour, J., Vinayagamoorhy, T., Jimbow, K., Chen, H., Luo, D., Dixon, W., and Munoz, V. (1993) Identification of a cDNA coding for Ca^{2+}-binding phosphoprotein (p90), Calnexin, on melanosome in normal and malignant human melanocytes. *Exp. Cell Res.* **209**, 288-300
17. Jimbow, K., Hara, H., Vinayagamoorthy, T., Luo, D., Dakour, J., Yamada, K., Dixon, W., and Chen, H. (1994) Molecular control of melanogenesis: Functional assessment of tyrosinase and Lamp gene families by UV exposure and gene co-transfection, and cloning of a cDNA encoding calnexin, a possible melanogenesis "chaperone." *J. Dermatol.* **21**, 894-906
18. Vinayagamoorthy, T., Dakour, J., Dixon, W., and Jimbow, K. (1993) cDNA-based functional domains of a calnexin-like melanosomal protein, p90. *Melanoma Res.* **3**, 463-469
19. Toyofuku, K., Park, J., Chen, H., and Jimbow, K. (1995) Tyrosinase requires a molecular chaperone, calnexin/p90 for efficient post-translational glycosylation and enzyme activity (abstract) (6th Annual Meeting of the Pan American Society for Pigment Cell Research (Kansas City, Missouri), June 25-28. *Pigment Cell. Res.* **4**, 17
20. Bergeron, J.J., Brenner, M.B., Thomas, D.Y., and Williams, D.B. (1994) Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* **19**, 124-128
21. Hammond, C., Braakman, J., and Helenius, A. (1994) Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA* **91**, 913-917
22. Wada, I., Kai, M., Imai, S., Sakane, F., and Konoh, H. (1997) Promotion of transferrin folding by Golic interactions with calnexin and calreticulin. *EMBO J.* **16**, 5420-5432
23. Ware, F.E., Vassilakos, A., Peterson, P.A., Jackson, M.R., Lehrman, M.A., and Williams, D.B. (1995) The molecular chaperone calnexin binds Glu1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J. Biol. Chem.* **270**, 4697-4704
24. Korner, A.M. and Pawelek, J.M. (1982) Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. *Science* **217**, 1163-1165
25. Hearing, V.J. (1987) Mammalian monophenol monooxygenase (tyrosinase): purification, properties, and reaction catalyzed. *Methods Enzymol.* **142**, 154-165
26. Jimbow, K., Quevedo, W.C., Fitzpatrick, T.B., and Szabo, G. (1993) Biology of melanocyte in *Dermatology in General Medicine* (Fitzpatrick, T.B., Eisen, A.Z., Wolff, K., Freedberg, I.M., and Austen, K.F., eds.) pp. 261-289, McGraw-Hill, New York
27. Imokawa, G. and Mishima, Y. (1982) Loss of melanogenetic properties in tyrosinase induced by glyCOSylation inhibitor within malignant melanoma cells. *Cancer Res.* **42**, 1994-2002
28. Jimenz, M., Tsukamoto, K., and Hearing, V.J. (1991) Tyrosinase from two different loci are expressed by normal and transfected melanocytes. *J. Biol. Chem.* **266**, 1147-1156
29. Rajagopalan, S., Xu, Y., and Brenner, M.B. (1994) Retension of unassembled component of integral membrane proteins by calnexin. *Science* **263**, 387-390
30. Chen, C. and Okayama, H. (1987) High-efficiency transfection of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752
31. Jimbow, K., Roth, S.I., Fitzpatrick, T.B., and Szabo, G. (1975) Mitotic activity in non-neoplastic melanocytes in vivo as determined by histochemical, auto-radiographic, and electron microscope studies. *J. Cell Biol.* **66**, 663-671
32. Alena, F., Jimbow, K., and Ito, S. (1990) Melanocytotoxicity and antimelanoma effects of phenolic amine compounds in mice *in vivo*. *Cancer Res.* **50**, 3743-3747
33. Lee, T.H., Lee, M.S., and Lu, M-Y. (1998) Effects of α-MSH on melanogenesis and tyrosinase of B-16 melanoma. *Endocrinology* **91**, 1180-1188
34. Odorigzi, G., Cowles, C.R., and Emv, S.D. (1998) The AP-3 complex: a coat of many colors. *Trends Cell Biol.* **8**, 282-288
35. Hammond, C. and Helenius, A. (1994) Folding of VSV G protein: Sequential interaction with Bip and calnexin. *Science* **266**, 456-458
36. Bouchard, B., Fuller, B.B., Vijayasaradhi, S., and Houghton, A.N. (1989) Induction of pigmentation in mouse fibroblast by expression of human tyrosinase cDNA. *J. Exp. Med.* **169**, 2029-2042
37. Jackson, M.R., Cohen-Doyle, M.F., Peterson, P.A., and Williams, D.B. (1994) Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science* **263**, 384-387
38. Zhang, Q., Tector, M., and Salter, R.D. (1995) Calnexin recognizes carbohydrate and protein determination of class I major histocompatibility complex molecules. *J. Biol. Chem.* **270**, 3944-3948
39. Gelman, M.S., Chang, W., Thomas, D.Y., Bergeron, J.J.M., and Prives, J.M. (1995) Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of nicotinic acetylcholine receptor. *J. Biol. Chem.* **270**, 15085-15092
40. Tector, M. and Salter, R.D. (1995) Calnexin influences folding on human class I histocompatibility proteins but not their assembly with beta-2 microglobulin. *J. Biol. Chem.* **270**, 19638-19642
41. Hebert, D.N., Foellmer, B., and Helenius, A. (1996) Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. *EMBO J.* **12**, 2961-2968

42. Ora, A. and Helenius, A. (1995) Calnexin fails to associate with substrate proteins in glucosidase-deficient cell lines. *J. Biol. Chem.* **270**, 26060-26062
43. Balow, J.P., Weissman, J.D., and Kearse, K.P. (1995) Unique expression of major histocompatibility complex class I proteins in the absence of glucose trimming and calnexin association. *J. Biol. Chem.* **270**, 29025-29029
44. Chen, W., Helenius, J., Braakmanl, I., and Helenius, A. (1995) Cotranslational folding and calnexin binding of influenza hemagglutinin in the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **92**, 6229-6233
45. Watowich, S.S. and Morimoto, R.I. (1988) Complex regulation of heat shock- and glucose-responsive genes in human cells. *Mol. Cell Biol.* **8**, 393-405
46. Windhorst, D.B., Zelickson, A.S., and Good, R.A. (1966) Chediak Higashi syndrome: hereditary gigantism of cytoplasmic organelles. *Science* **151**, 81-83
47. Zelickson, A.S., Windhorst, D.B., White, J.G., and Good, R.A. (1967) The Chediak Higashi syndrome: formation of giant melanosomes and the basis of hypopigmentation. *J. Invest. Dermatol.* **49**, 575-581
48. Witkop, C. Jr., Wolfe, L.S., Cal, S.X., White, J.G., Townsend, D., and Keenan, K.M. (1987) Elevated urinary dolichol excretion in the Hermansky-Pudlak syndrome. Indicator of lysosomal dysfunction. *Am. J. Med.* **82**, 463-470
49. Stevenson, R.E., Lubinsky, M., Taylor, H.A., Wenger, D.A., Schroer, R.J., and Olmstead, P.M. (1983) Sialic acid storage disease with sialuria: chemical and biochemical features in the severe infantile type. *Pediatrics* **72**, 441-449
50. Pueschel, S.M., O'Shea, P.A., Alroy, J., Amber, M.W., Dandond, F., Daniel, P.F., and Kolodny, E.H. (1988) Infantile sialic acid storage disease associated with renal disease. *Pediatr. Neurol.* **4**, 207-211
51. Vijayasaradhi, S., Xu, Y., Bouchard, B., and Houghton, A.N. (1995) Intracellular orting and targeting of melanosomal membrane proteins: identification of signals for soaring of human brown locus protein, gp75. *J. Cell Biol.* **130**, 807-820