THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 28, Issue of July 11, pp. 25607–25617, 2003
Printed in U.S.A.

Tyrosinase Maturation and Oligomerization in the Endoplasmic Reticulum Require a Melanocyte-specific Factor*

Received for publication, April 2, 2003, and in revised form, April 30, 2003
Published, JBC Papers in Press, April 30, 2003, DOI 10.1074/jbc.M303411200

Edwin Francis‡, Ning Wang‡, Hadas Parag‡, Ruth Halaban§, and Daniel N. Hebert‡¶

From the ‡Department of Biochemistry and Molecular Biology, Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003 and §Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut 06520

Tyrosinase is a glycoprotein responsible for the synthesis of melanin in melanocytes. A large number of mutations have been identified in tyrosinase, with many leading to its misfolding, endoplasmic reticulum (ER) retention, and degradation. Here we describe the folding and maturation of human tyrosinase (TYR) using an *in vitro* translation system coupled with ER-derived microsomes or with semipermeabilized cells, as an intact ER source. TYR remained misfolded as determined by its sensitivity to trypsin digestion and its persistent interaction with the ER resident lectin chaperones calnexin and calreticulin when produced in ER-derived microsomes or nonmelanocytic semipermeabilized cells. However, when TYR was translocated into semipermeabilized melanocytes, chaperone interactions were transient, maturation progressed to a trypsin-resistant state, and a TYR homodimer was formed. The use of semipermeabilized mouse melanocytes defective for tyrosinase or other melanocyte-specific proteins as the ER source indicated that proper TYR maturation and oligomerization were greatly aided by the presence of wild type tyrosinase and tyrosinase-related protein 1. These findings suggested that oligomerization is a step in proper TYR maturation within the ER that requires melanocyte-specific factors.

Tyrosinase (monophenol monooxygenase; EC 1.14.18.1) catalyzes the rate-limiting oxidation step in melanin production (1). This melanocyte-specific type I membrane glycoprotein is localized to the post-Golgi compartment termed the melanosome (for review, see Ref. 2). Mutations in tyrosinase are the cause of oculocutaneous albinism type 1 (3). Mutant tyrosinase is recognized as a misfolded protein by the quality control system of the endoplasmic reticulum (ER),[1] where it is targeted for retention (4–6). In addition, wild type (WT) tyrosinase in amelanotic melanoma cells is retained in the ER in response to an acidic environment characteristic of tumor cells and is eventually subjected to ER-associated protein degradation (7, 8). Tyrosinase has been used as a model substrate to study glycoprotein maturation in the secretory pathway because of its association with human inherited conditions and the ease in assessing its enzymatic activity by monitoring the production of melanin.

Proteins that traverse the eukaryotic secretory pathway, such as tyrosinase, are generally co-translationally inserted through a translocon located within the ER membrane (9). Upon emergence of the polypeptide chain into the ER lumen, the protein is immersed into a specialized maturation environment. The oxidizing conditions of the ER support protein-assisted disulfide bond formation (10). Additional ER protein-mediated maturation processes include signal sequence cleavage, protein folding, glycan transfer and trimming, chaperone binding, and oligomerization (11). Understanding these maturation steps has implications for the etiology of albinism, the production of antigenic peptides associated with melanoma, and the wide array of other ER retention diseases.

Human tyrosinase (TYR) contains seven *N*-linked glycosylation sites. Although all seven sites are utilized, one site (Asn^{290}) is inefficiently processed due to an adjacent Pro residue (Asn-Gly-Thr-Pro) (12). The presence of this inefficient glycosylation site, which is absent in mouse tyrosinase (Tyr), creates a heterogeneous population of TYR possessing six or seven glycans. Glycan trimming by glucosidases I and II generates monoglucosylated side chains, which are, in turn, substrates for calnexin (CNX) and calreticulin (CRT), ER lectin chaperones that specifically bind monoglucosylated glycans (13, 14). The interaction of CNX and CRT with TYR appears to be essential for its proper maturation and the acquisition of its activity within the cell. Inhibition of glucose trimming in human melanoma cells decreased TYR activity and stability (15). In addition, inactive tyrosinase was produced in B16 mouse melanoma cells treated with glucosidase inhibitors (16, 17). The removal of the final glucose by glucosidase II triggers the release of TYR from CNX and CRT. Reglucosylation by the UDP-glucose:glycoprotein glucosyltransferase, which reglucosylates proteins containing non-native regions (18), can initiate rebinding of the substrates to the lectin chaperones (19).

In this study, a semipermeabilized melanocyte system coupled with *in vitro* translations that supported the proper oxidation, chaperone binding, and oligomerization of TYR was developed. This system provides a high degree of experimental versatility, as well as the ability to isolate and identify the ER components required for TYR maturation. We found that TYR matured to a newly defined homodimer after oxidation and release from CNX. This oligomerization required melanocyte-specific factors.

* This work was supported by United States Public Health Service Grants CA79864 (to D. N. H.), CA44542 (to R. H.), and AR41942 (to Yale Skin Diseases Research Center; R. E. Tigelaar, Program Investigator). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 413-545-0079; Fax: 413-545-3291; E-mail: dhebert@biochem.umass.edu.

[1] The abbreviations used are: ER, endoplasmic reticulum; BMH, bismaleimidohexane; Tyrp, tyrosinase-related protein; TYR, human tyrosinase; Tyr, mouse tyrosinase; WT, wild type; CNX, calnexin; CRT, calreticulin; RRL, rabbit reticulocyte lysate; WG, wheat germ; CHO, Chinese hamster ovary; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

MATERIALS AND METHODS

Reagents—Flexi rabbit reticulocyte lysate (RRL), wheat germ (WG), dithiothreitol, and RNasin were from Promega. EasyTag [^{35}S]methionine/cysteine and restriction endonucleases were from PerkinElmer Life Sciences and New England Biolabs, Inc. (Beverly, MA), respectively. The T7 transcription kit was from Ambion (Austin, TX). Antibodies against calreticulin (PA3-900; rabbit) and TYR (M-19; goat) were from Affinity BioReagents (Golden, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other reagents were from Sigma.

Cell Lines—Wild type black and c-albino mutant (Tyr(C85S)) mouse melanocytes were maintained in culture as described previously (4). Mouse melanocytes expressing the null mutant pink eye dilution protein p^{cp}/p^{25H} (melan-p) (20) or mutant tyrosinase-related protein 1 (Tyrp1(C86Y)) (melan-b) (21, 22) were established in culture by Dr. D. C. Bennett (Department of Anatomy, University of London, London, United Kingdom) and obtained from Dr. V. Hearing (Laboratory of Cell Biology, National Institutes of Health, Bethesda, MD). The melanoma cells 501 mel and YUGEN8 were grown in Ham's F-10 medium as described previously (8). Chinese hamster ovary (CHO) cells were grown in α-MEM in 10% fetal bovine serum.

Construction of Plasmids Encoding Wild Type and Mutant TYR—The construction of the plasmid encoding WT TYR, termed pSP72-K^bSS/TYR, has been described previously (12). To generate the mutant TYR forms C89R and C500S, the corresponding Cys in pSP72-K^bSS/TYR were changed to Arg and Ser, respectively, using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The truncated tail-less TYR (ΔTail) was created by the insertion of a stop codon in pSP72-K^bSS/TYR at position 499 of the protein. All mutations were verified by DNA sequencing.

Transcription, Translation, and Translocation—Messenger RNA was prepared by *in vitro* run-off transcription after linearizing the TYR-encoding plasmid with *Nde*I restriction enzyme. Radioactive ^{35}S-labeled TYR was translated for 1 h at 27 °C with RRL or WG in the presence of canine pancreas microsomes or semipermeabilized cells. Translation reactions were carried out as described previously (12). In some experiments, the samples were alkylated either with *N*-ethylmaleimide (20 mM) (23) or with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; 14 mM) in 80 mM Tris, pH 6.8, 1% SDS to block free sulfhydryls. Lysates of alkylated samples were analyzed directly on SDS-PAGE or after immunoprecipitation with various antibodies before electrophoresis. In most experiments, one half of the sample was subjected to nonreducing SDS-PAGE, and the other half was reduced by the addition of dithiothreitol (100 mM) before electrophoresis.

Preparation of Semipermeabilized Cells—Semipermeabilized cells were prepared from confluent cultures as described previously (24). In brief, confluent cells (~5×10^7 cells) were detached from the culture dish (75 cm^2) with 4 ml of a solution of 0.25% trypsin/EDTA and collected in 8 ml of KHM buffer (110 mM KOAc, 2 mM MgOAc, 20 mM HEPES, pH 7.2). Cells were harvested by centrifugation at 1,200 rpm for 7 min at 4 °C. Cell pellets were resuspended in KHM buffer (6 ml) and permeabilized with digitonin (20 μg/ml) on ice for 5 min. Permeabilization was stopped by the addition of KHM buffer (8 ml) followed by centrifugation. Cell pellets were resuspended in resuspension buffer (14 ml; 50 mM KOAc, 90 mM HEPES, pH 7.2), incubated on ice for 10 min, and centrifuged at 1,200 rpm for 7 min at 0 °C. Pellets were resuspended in KHM buffer (~4×10^6 cells/100 μl KHM) and treated with a calcium-dependent nuclease at 25 °C for 12 min to remove the endogenous mRNAs. Nuclease treatment was stopped by EGTA (final concentration, 4 mM). The cells were centrifuged at 8,000 rpm for 5 min at 4 °C, and cell pellets were resuspended in KHM buffer to a final concentration of 1×10^5 cells/μl. Radioactively labeled TYR was translated for 1 h at 27 °C in RRL with semipermeabilized cells (1.3×10^4 cells/μl) substituting for the rough ER-derived microsomes.

Sucrose Density Gradient Centrifugation—To determine the size of the monomeric and oligomeric TYR generated with semipermeabilized cell system, ^{35}S-labeled samples were lysed in 2% CHAPS in 90 mM HEPES and 50 mM NaCl (HBS), pH 7.5. Cell debris were removed by centrifugation at 14,000 rpm in a microfuge at 4 °C for 5 min, and the supernatant (150 μl) was layered on top of a 5–25% linear sucrose gradient (4 ml) in HBS supplemented with 0.5% CHAPS, pH 7.5. The gradients were centrifuged at ~197,600 × *g* in a Beckman (Model L80-M) ultracentrifuge for 15 h at 4 °C; fractions were collected manually, subjected to immunoprecipitation with anti-TYR antibodies, and analyzed by SDS-PAGE.

Trypsin Digestion—Radioactively labeled *in vitro*-translated TYR was treated with trypsin to assess its stability. After increasing maturation times, samples were removed, and free thiols were alkylated with *N*-ethylmaleimide. ER-translocated TYR was isolated by centrifugation at 14,000 rpm for 5 min at 4 °C. Microsome or semipermeabilized cell pellets were solubilized in 1% CHAPS/HBS (25 μl/10 μl translation mixture) and divided into two fractions. Trypsin was added to one set of samples (1–3 ng/μl), followed by incubation at 27 °C for 10 min. The digestions were stopped with soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (4 mM).

Chemical Cross-linking—To identify the TYR oligomer, WT Tyr or mutant (TyrC85S) albino mouse melanocytes were suspended in phosphate-buffered saline (1–3 × 10^6), and half of the samples were subjected to chemical cross-linking with bismaleimidohexane (BMH; 1 mM) at 25 °C for 30 min followed by blocking with 2-mercaptoethanol (10 mM). After centrifugation, cell pellets were lysed with 2% CHAPS/HBS supplemented with protease inhibitors, cell lysates were cleared from debris by centrifugation, and supernatants were subjected to Western blotting with anti-tyrosinase M-19 goat polyclonal antibodies (Santa Cruz Biotechnology).

RESULTS

Defective TYR Maturation in Rough ER Microsomes Derived from Pancreatic Acinar Cells—A cell-free system was used to characterize the maturation of TYR in the early secretory pathway. We have shown previously that ER translocation and co-translational glycosylation of TYR can be monitored in a cell-free system composed of an *in vitro* translation system coupled with canine pancreas ER-derived microsomes (12). In these studies, replacing the native signal sequence of TYR with that of the murine major histocompatibility complex class I molecule K^b (K^bSS) increased the efficiency of translation and translocation by severalfold. Therefore, this construct, termed pSP72-K^bSS/TYR, has been used throughout the following studies and is referred to as WT TYR.

To monitor the oxidation of TYR in the ER, WT TYR was translated in RRL or WG translation systems supplemented with rough ER-derived microsomes. After 1 h of translation, protein synthesis was arrested with cycloheximide, and the protein was allowed to mature for increasing periods of time (Fig. 1*A*). The oxidative state of TYR was trapped with the alkylating agent *N*-ethylmaleimide and analyzed directly (*Lysate*) or after immunoprecipitation with anti-tyrosinase antibodies (*α-TYR*) by nonreducing and reducing SDS-PAGE.

ER-translocated and *N*-linked glycosylated TYR (TYR) appeared as a 70-kDa doublet upon reduction (Fig. 1*A*, *bottom panel*). The doublet corresponds to TYR possessing all seven (*TYR*^{7}) or six (*TYR*^{6}) *N*-linked glycans (12). As reported previously, the fraction of TYR^6 was increased in the faster-translating RRL system compared with the slower WG translation, where each form was present in equal amounts. In the nonreduced (*NR*) samples, TYR displayed a slight increase in mobility indicative of intramolecular disulfide formation creating a more compact structure (Fig. 1*A*, *top panel*). TYR aggregates were also observed at the top of the nonreduced gel. These aggregates disappeared upon reduction, demonstrating that they involved intermolecular disulfide bonds. An artifact of the cell-free maturation system is the generation of untranslocated protein (*^{U}TYR*) that is not properly targeted to the ER microsomes. Therefore, translated TYR also accumulates as unglycosylated protein possessing an intact N-terminal signal sequence with an electrophoretic mobility of ~60 kDa (Fig. 1*A*, *bottom panel*). The untranslocated protein accounts for a portion of the aggregates observed upon oxidation.

TYR has 15 lumenal Cys that can potentially form multiple intra- and/or intermolecular disulfide bonds. Because the mobility shift observed under nonreduced conditions was slight, the status of the Cys thiols upon oxidation was determined by modification of free thiols with a bulky membrane-impermeable alkylating agent, AMS (536 Da). Radioactively labeled TYR was translated in RRL or in WG in the presence of ER-derived microsomes under reducing or oxidizing conditions,

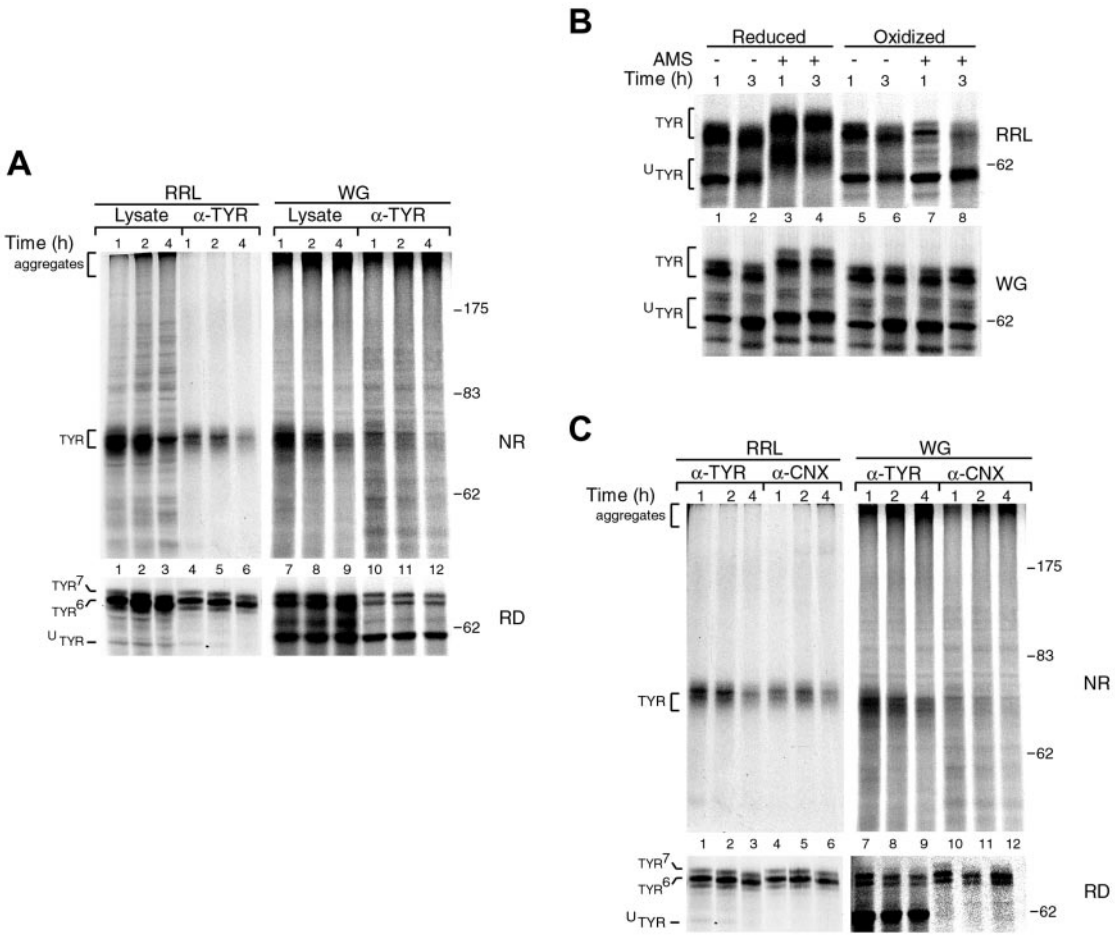

FIG. 1. **TYR oxidation in RER microsomes.** *A*, wild type TYR was translated in RRL or in a WG translation system in the presence of canine pancreas microsomes for 1 h and allowed to mature further for the times indicated. Half of the lysate was immunoprecipitated with anti-TYR (*α-TYR*) antibodies, and samples were resolved by SDS-PAGE under reducing (*RD*; *bottom panel*) and nonreducing (*NR*; *top panel*) conditions and visualized by autoradiography. *B*, the status of free Cys in TYR was analyzed by treating the lysates after 1-h translation under reducing and oxidizing conditions with (+) or without (−) AMS. Samples were resolved by reducing SDS-PAGE. *C*, CNX binding to WT TYR was monitored up to 4 h of maturation in the presence of microsomes by the co-immunoprecipitation of ^{35}S-TYR with anti-CNX antibodies (*α-CNX*). Samples were separated on reducing and nonreducing SDS-PAGE. In this figure and all others, TYR^7 and TYR^6 denote translocated and glycosylated TYR with seven and six *N*-linked glycans (12). Untranslocated and unglycosylated TYR is designated as ^{U}TYR. Molecular mass markers are indicated to the *right* of the gels in kDa.

followed by alkylation with *N*-ethylmaleimide or AMS before SDS-PAGE analysis under reducing conditions. A large decrease in mobility of translocated and untranslocated TYR was observed when the protein was translated under reducing conditions, in which Cys residues are accessible to AMS (Fig. 1*B*, *lanes 1–4*). However, no mobility shift in TYR was seen under oxidizing conditions (Fig. 1*B*, *lanes 5–8*), indicating that most of the Cys residues were protected from alkylation, probably due to their involvement in disulfide bonds. Similar results were observed regardless of the cell-free translation system used (RRL or WG), indicating that TYR formed disulfide bonds in both conditions.

Calnexin Binding to TYR Persists in the RER Microsomes—Chaperones bind transiently to substrates until they acquire correct tertiary or quaternary structure. Similarly, TYR associates with two lectin chaperones, CNX and CRT, before exit from the ER (7). We therefore determined whether TYR was maturing correctly in ER-derived microsomes by monitoring complex formation with CNX. Immunoprecipitation of radioactively labeled TYR with anti-TYR (*α-TYR*) or anti-CNX (*α-CNX*) antibodies showed that TYR interaction with CNX persisted even after 4 h of processing, regardless of the cell-free system used (Fig. 1*C*, *lanes 4–6* and *10–12*). Persistent interactions were similarly observed for CRT, the soluble lumenal paralogue of CNX (data not shown). Therefore, unlike other membrane glycoproteins such as influenza hemagglutinin and vesicular stomatitis virus G protein (14, 25), the canine pancreatic microsomes cell-free system does not support the proper maturation of the type I membrane glycoprotein TYR.

TYR Matures Efficiently in Semipermeabilized Melanocytes—Since TYR is a melanocyte-specific protein, it is possible that a factor specific to melanocytes is required for its proper maturation. To address this concern, we used a semipermeabilized cell system that can be coupled to the *in vitro* translation of a substrate (26). Treatment of mouse melanocytes with digitonin produced semipermeabilized cells with a leaky plasma membrane but an intact ER as confirmed by confocal immunofluorescence microscopy (data not shown). We have previously found that this semipermeabilized melanocyte system supports the efficient ER translocation and glycosylation of TYR (12).

In vitro-translated TYR translocated into the ER of wild type semipermeabilized melanocytes was analyzed directly or after immunoprecipitation with anti-TYR or anti-CNX antibodies. As previously observed in microsomes, TYR translocated into the ER from semipermeabilized cells migrated as a doublet (Fig. 2*A*, *lane 1*). TYR migrated faster in SDS-PAGE under nonreduced (*NR*) compared with reduced (*RD*) conditions after 2 and 4 h of maturation, indicative of disulfide bond formation

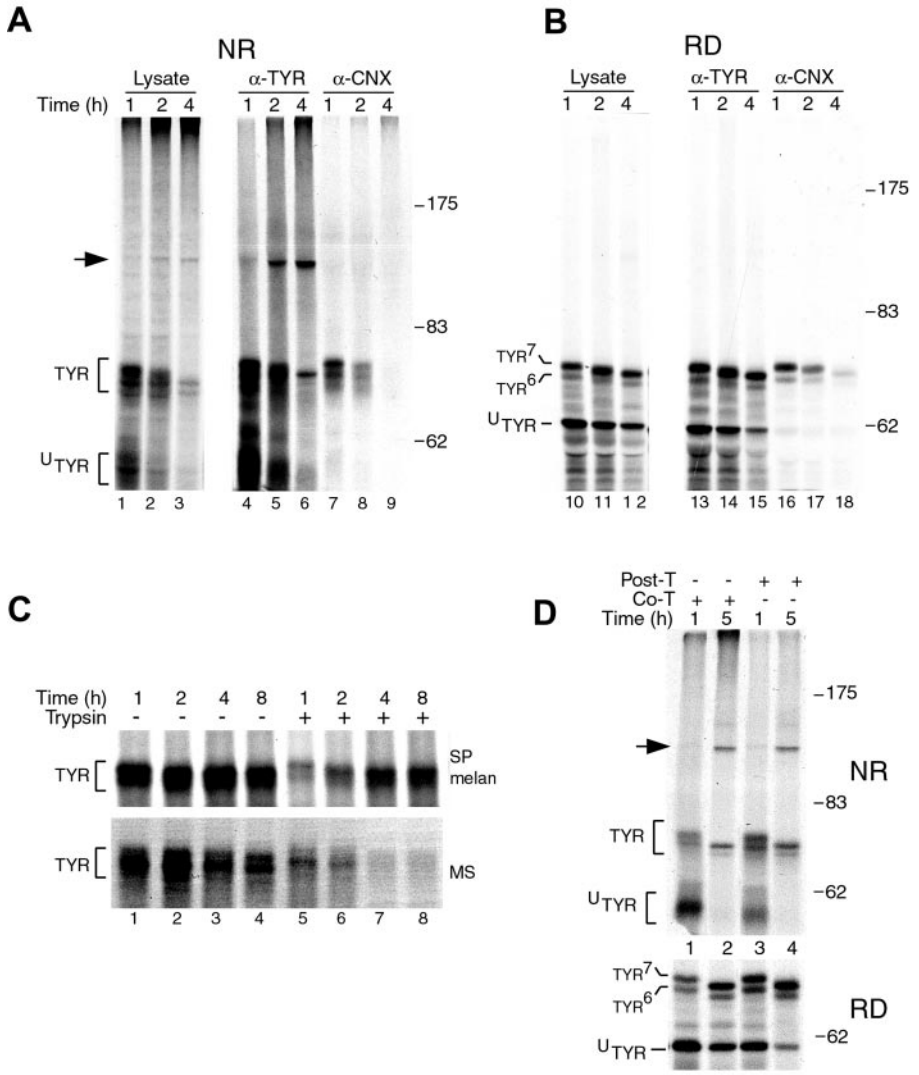

FIG. 2. **TYR matures and oligomerizes in semipermeabilized wild type melanocytes.** WT TYR translated in rabbit reticulocyte lysate in the presence of semipermeabilzied wild type mouse melanocytes was allowed to mature for increasing periods of time. Samples were immunoprecipitated with anti-TYR or anti-CNX antibodies. Lysates and immunoprecipitates were visualized by autoradiography after nonreducing (*NR*; *A*) and reducing (*RD*; *B*) SDS-PAGE. *C*, TYR was translated under oxidizing condition for the indicated times in the presence of microsomes (*MS*) or semipermeabilized melanocytes (*SP melan*). Translocated proteins were separated by centrifugation of the membranes as described under "Materials and Methods." Solubilized membrane pellets were treated with trypsin at 25 °C for 10 min, and reduced samples were resolved on a 7.5% SDS-PAGE and visualized by autoradiography. *D*, TYR oxidation was initiated during translation (co-translationally, *Co-T*; *lanes 1* and *2*) or post-translationally (*Post-T*; *lanes 3* and *4*) by the addition of FAD. Radioactive TYR was resolved by nonreducing and reducing SDS-PAGE. The *arrow* indicates TYR oligomers.

(Fig. 2, compare *A* with *B*). Interestingly, a disulfide-linked oligomer band of 140 kDa appeared after 2 h of maturation, increasing in intensity with time (Fig. 2*A*, *arrow*). This oligomer disappeared after reduction, suggesting that it was created by intermolecular disulfide bonds (Fig. 2*B*).

Evidence for proper maturation in semipermeabilized melanocytes was provided by the transient binding of CNX to TYR. The CNX·TYR complex observed during the first 2 h of processing was greatly diminished at the end of 4 h of maturation (Fig. 2*A*, *lane 9*). Furthermore, the 140-kDa TYR oligomer was not bound by CNX. The temporal relationship indicated that TYR oligomerization occurred after CNX release and is a properly folded form because CNX is released from monoglucosylated substrates once the substrate is trimmed of its final glucose and is no longer reglucosylated by UDP-glucose:glycoprotein glucosyltransferase (13, 14, 19). The time-dependent accumulation of trimmed or unglucosylated TYR side chains was supported by the persistence of an increase in TYR mobility observed after reduction with dithiothreitol (Fig. 2*B*, *lanes 10–15*).

Resistance to limited proteolysis was used as an additional measure for TYR maturation because folded proteins acquire a more stable conformation that is often resistant to digestion by proteases. The vast majority of TYR produced in the RRL microsome system under oxidizing conditions that permit the formation of disulfide bonds remained protease-sensitive, even after 8 h of maturation (Fig. 2*C*, *bottom panel*, *lanes 5–8*). In contrast, a substantial level of trypsin-resistant TYR was already present after 4 h of maturation, a time at which CNX no longer bound to TYR. Therefore, semipermeabilized melanocytes appear to support the proper folding of TYR in the ER.

Folding and disulfide bond formation commence co-translationally in the ER (27). To determine whether TYR could form oligomers when proper oxidizing conditions were initiated post-translationally, ^{35}S-TYR was translated for 1 h under reducing condition, and the oxidizing agent FAD was added after inhibition of further protein synthesis with cycloheximide (Fig. 2*D*). Oligomerization of TYR was observed after 4 h of post-translational oxidation, indicating that the physiological co-translational maturation process was not obligatory for oligomerization in semipermeabilized melanocytes (Fig. 2*D*, *top panel*, *lane 4*).

Time-dependent Oligomerization of TYR—To further characterize the oligomeric state, ^{35}S-TYR generated in the presence of WT semipermeabilized mouse melanocytes was solubilized with the nondenaturing detergent CHAPS and subjected to centrifugation through a 5–25% linear sucrose gradient. After 1 h of processing, TYR was predominantly in the ~5S form (Fig. 3, *lanes 6* and *7*, *1hr*) corresponding to monomeric TYR. An additional smaller peak in *lanes 8–10* was indicative of oligomeric TYR of ~8S. The quaternary structure of the 8S form of TYR was disrupted by an ionic detergent because it migrated as a monomer of 70 kDa on SDS-PAGE. The 8S isoform likely represented TYR heterocomplexes with CNX

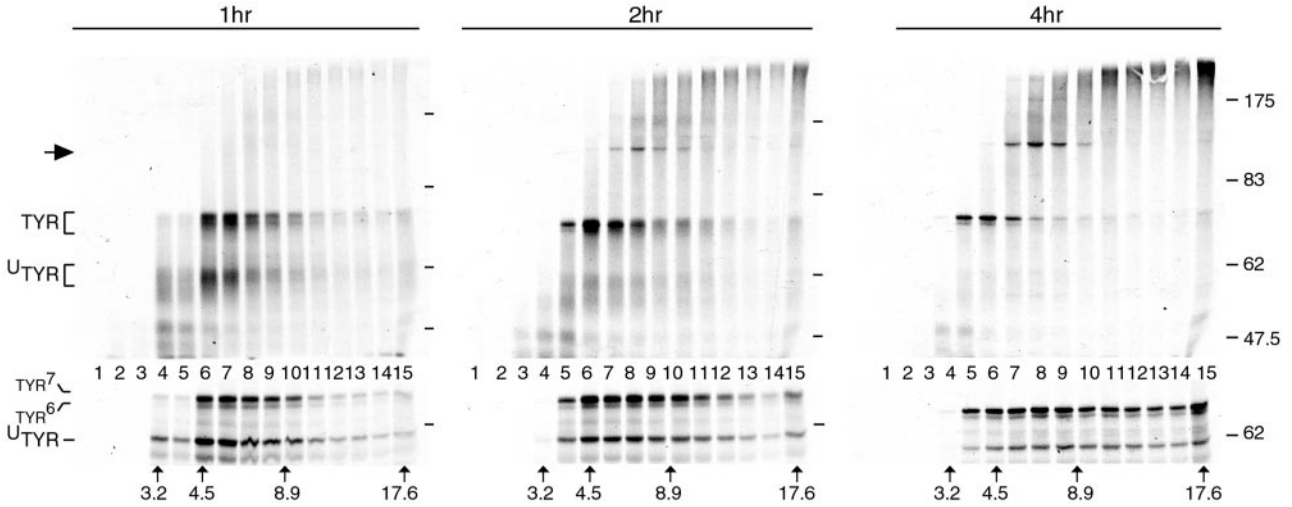

FIG. 3. **Characterization of TYR oligomer by sucrose density gradient ultracentrifugation.** Radioactively labeled tyrosinase samples were lysed in 2% CHAPS and separated on a 5–25% linear sucrose gradient by ultracentrifugation. TYR was immunoprecipitated with anti-TYR antibodies before separation by nonreducing and reducing SDS-PAGE.

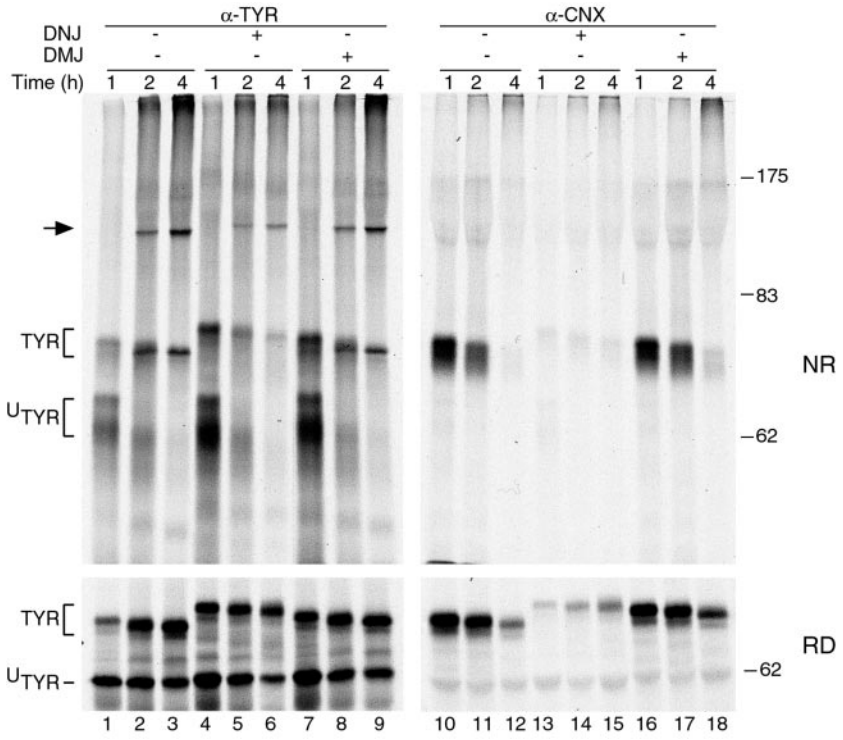

FIG. 4. **TYR oligomerization does not require glycan trimming.** TYR was translated in the presence of semipermeabilized melanocytes for up to 4 h in the absence or presence of glucosidase (deoxynojirimycin, *DNJ*) or mannosidase (deoxymannojirimycin, *DMJ*) inhibitors. Radioactively labeled TYR was subjected to immunoprecipitation with anti-TYR (*α-TYR*) or anti-CNX (*α-CNX*) antibodies, and products were resolved by reducing and nonreducing SDS-PAGE.

and/or CRT because the interaction is stable in CHAPS but not in SDS-containing buffer (28).

After further maturation for 2 and 4 h, the monomeric peak moved from *lane 7* to *lane 6*, indicative of a decline in CNX and CRT binding to TYR. In addition, a 7.5 S peak that was resolved as ~150 kDa by SDS-PAGE emerged (Fig. 3, *2hr* and *4hr*, *arrow*). The intensity of this peak increased with time, as did the aggregated form of TYR, which accumulated at the top of the gel. Both velocity centrifugation and SDS-PAGE analyses indicated that the 150-kDa oligomeric form of TYR entails the addition of 70–80 kDa of molecular mass.

TYR Oligomerization Does Not Require Glycan Trimming—To determine the roles of glycosidase trimming and subsequent CNX and CRT binding in TYR oligomerization, the maturation of TYR in semipermeabilized melanocytes was monitored in the presence of *n*-butyl deoxynojirimycin and deoxymannojirimycin, inhibitors of ER glucosidases and mannosidases, respectively. Inhibition of mannose trimming had no effect on CNX binding or TYR oligomerization (Fig. 4, *lanes 7–9* and *16–18*). As expected, inhibition of glucose trimming resulted in a slower-migrating protein due to the persistence of triglucosylated side chains (Fig. 4, compare *lane 4* with *lane 1*, *NR* and *RD*) that were not bound by CNX (Fig. 4, *lanes 13–15*). However, TYR did oligomerize in the presence of the glucosidase inhibitor *n*-butyl deoxynojirimycin, although with less efficiency than in the absence of inhibitors (35% *versus* 50%, respectively). Therefore, CNX binding enhanced the efficiency of oligomerization but was not obligatory for this process.

Inactive Mutant TYR Does Not Form Oligomers—Mutations in tyrosinase are the cause of oculocutaneous albinism type 1, a recessive genetic disorder manifested by the absence of melanin in melanocytes (3). We therefore analyzed several mutant

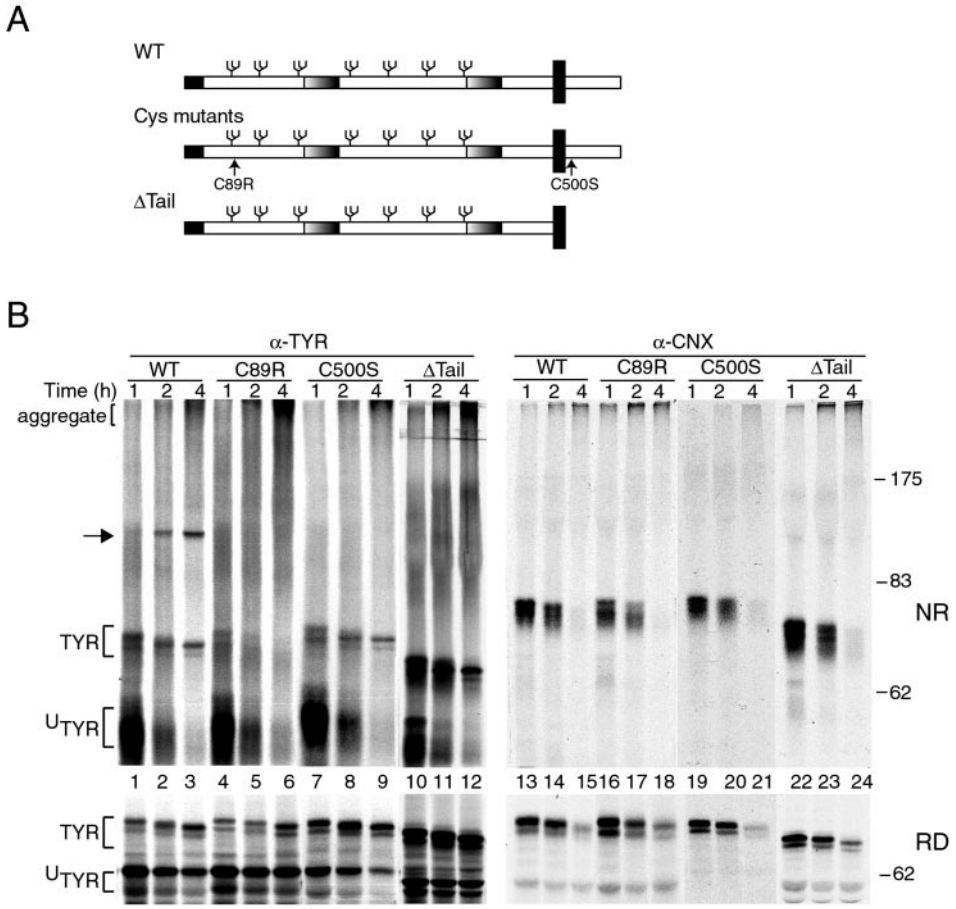

FIG. 5. **Mutations in TYR disrupt its oligomerization.** *A*, schematic representation of WT and mutant forms of TYR. Signal sequences and Cu binding sites are indicated by *solid* and *shaded boxes*, respectively. *Branched structures* denote *N*-link glycosylation sites. *B*, WT and mutant TYR translated in the presence of wild type semipermeabilized melanocytes were allowed to mature. Samples were aliquoted at the indicated times and immunoprecipitated with anti-TYR or anti-CNX antibodies. Proteins were visualized by autoradiography after reducing and nonreducing SDS-PAGE.

forms of TYR (described in Fig. 5*A*) to determine whether proper folding is required for oligomerization and to identify the amino acid residue responsible for intermolecular bonding. The inactive mouse mutant Tyr(C85S) in the albino Balb c mouse is retained in the ER as an immature 70-kDa glycoform bound to the ER chaperones CNX and CRT (4). The mutant protein is unstable, with a half-life of ~3 h, and is rapidly degraded by the ER protein-associated degradation pathway.[2] The homologous mutation in humans, TYR(C89R), is also associated with albinism (Fig. 5*A*, *Cys mutants*). TYR(C89R) was therefore used to determine whether inactive mutant TYR could also form oligomers. Time course maturation analysis failed to detect oligomeric forms of TYR(C89R) even at the end of the 4-h reaction time (Fig. 5*B*, *lanes 4–6*). However, a larger amount of TYR(C89R) aggregated at the top of the nonreducing gel when compared with WT TYR. Furthermore, CNX binding to the mutant TYR(C89R) was transient in a fashion similar to that of WT protein (Fig. 5*B*, *lanes 16–18*). In contrast to what had been observed in intact albino mouse melanocytes,[2] TYR(C89R) was eventually released from CNX, but it was not degraded because the proteasome is inhibited in the semipermeabilized melanocyte system by the hemin from the reticulocyte lysate.

Residue Cys^{500} in the Cytosolic Tail Is Required for TYR Oligomerization—An intermolecular disulfide bond appears to be involved in the formation of oligomeric TYR because it was disrupted under reducing conditions. To identify the Cys residues engaged in this process, we first determined whether the disulfide bond resided in TYR ectodomain or cytosolic tail by analyzing oligomerization of a tailless TYR possessing an intact transmembrane domain (Fig. 5*A*, Δ*Tail*). Time course maturation analysis implicated the TYR cytosolic tail in the oligomerization process because TYR ΔTail was unable to form oligomers (Fig. 5*B*, *lanes 10–12*), although it achieved proper folding as indicated by the timely release from CNX (*lanes 22–24*).

The cytosolic tail of TYR has a single Cys residue at position 500 immediately at the interface between the predicted membrane and transmembrane regions (Fig. 5*A*, *Cys mutants*). To determine whether this Cys residue was required for oligomerization, we monitored the maturation of TYR(C500S), a mutant in which Cys^{500} was changed to a Ser. Similar to the TYR tail truncation, TYR(C500S) was released from CNX but did not oligomerize (Fig. 5*B*, *lanes 19–21* and *7–9*), indicating that this Cys was involved in TYR oligomer formation.

WT TYR Oligomerization Does Not Occur in Tyr(C85S) Albino Melanocytes or CHO Cells—Mutations in tyrosinase can cause the retention of an inactive protein in the ER that is eventually degraded by the cytosolic proteasome (4).[2] In addition, human amelanotic melanoma cells have been shown to possess a hostile acidic environment that does not support proper maturation of wild type TYR (7, 8, 29). To establish the requirements for TYR oligomer formation, the maturation of

[2] S. Svedine, T. Wang, R. Halaban, and D. N. Hebert, manuscript in preparation.

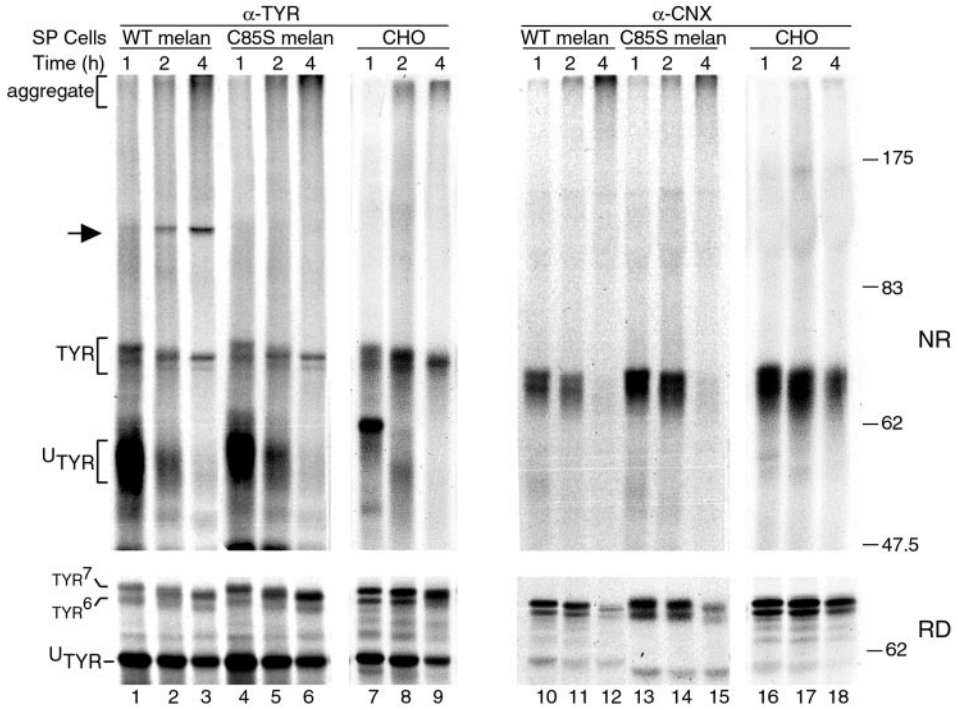

FIG. 6. **Oligomerization of TYR is cell type-dependent.** WT TYR was translated in rabbit reticulocyte lysate in the presence of semipermeabilized wild type or albino Tyr(C85S) melanocytes or CHO cells. Proteins were allowed to mature under oxidizing conditions, and samples were aliquoted at the indicated times for immunoprecipitation with anti-TYR or anti-CNX antibodies. Proteins were visualized by autoradiography after reducing and nonreducing SDS-PAGE. *Arrow* indicates the TYR oligomer band.

WT TYR was monitored in semipermeabilized mouse Tyr(C85S) albino melanocytes (4) and nonmelanocytic CHO cells. As before, oligomeric WT TYR appeared after 2 h of maturation in semipermeabilized wild type mouse melanocytes (Fig. 6, *lanes 2* and *3*, *top panel*), whereas the 150-kDa TYR oligomer was not observed when maturation proceeded in mouse Tyr(C85S) albino melanocytes or CHO semipermeabilized cells (Fig. 6, *lanes 4–9*).

Interaction of WT TYR with CNX during maturation in the different cell types was also examined. Co-immunoprecipitation experiments showed that CNX binding/release profiles in albino Tyr(C85S) melanocytes were similar to those in wild type melanocytes, even though TYR oligomerization was not observed (Fig. 6, *lanes 13–15*). In contrast, the interaction of CNX with WT TYR persisted when processed in the CHO semipermeabilized cells, indicating a failure to fold properly in this heterologous cell type. TYR also failed to oligomerize in semipermeabilized human amelanotic melanoma cells (501 mel and YUGEN8), although its binding to CNX was transient (7) (data not shown). Altogether, these results indicated that correct folding and maturation of TYR require factors associated with the ER that are not present or are nonfunctional in semipermeabilized cells lacking tyrosinase activity, such as the albino Tyr(C85S) melanocytes, amelanotic melanoma cells, or CHO cells.

Mouse Tyrp1 Is Required for Tyrosinase Oligomerization—Genetic analysis of inherited hypopigmentation disorders has demonstrated that mutations in other genes can modify the stability of tyrosinase and its subcellular localization and function (30–34). To determine whether defects in other melanocyte-specific proteins can also disrupt TYR oligomerization, TYR maturation was monitored in melanocytes expressing null p-protein mutant p^{cp}/p^{25H} (melan-p1) or Tyrp1(C86Y) (melan-b) (Fig. 7).

WT TYR oligomerization and CNX binding were normal when *in vitro* maturation was performed in semipermeabilized melan-p1 melanocytes, indicating that the p-protein was dispensable for these two processes (Fig. 7*A*, *lanes 4–6* and *13–15*). In contrast, WT TYR did not oligomerize when processed in semipermeabilized melan-b melanocytes (Fig. 7*B*, *lanes 4–6*); however, it was released from CNX (Fig. 7*B*, *lanes 10–12*).

Oligomerization Can Occur in Intact Melanocytes—Eukaryotic cells maintain redox conditions where the ER lumen provides an oxidizing environment, and the cytosol provides a reducing environment. Therefore, disulfide bond formation, for the most part, occurs in the ER, with the help of dedicated folding catalysts (10). Whereas disulfide bonds can be found in the cytosol, they are uncommon. The partitioning between oxidizing and reducing environments is not fully recapitulated in the *in vitro* translation and semipermeabilized cell systems. Here, the redox conditions are controlled by the reticulocyte lysate, in which FAD facilitates an oxidizing environment not only in the ER but also on the *cis* side of the ER membrane, where the Cys^{500} residue of TYR is localized. Therefore, to ensure that the oligomers observed with semipermeabilized melanocytes also occur in live cells under physiological conditions, we used the membrane-permeable chemical cross-linker BMH, a reagent that reacts with free Cys residues, to trap potential oligomers.

Wild type and Tyr(C85S) albino melanocytes were treated with BMH, and the cross-linker was then quenched with 2-mercaptoethanol before cell lysis. A tyrosinase oligomer of ~150 kDa was formed after cross-linking in wild type but not albino Tyr(C85S) melanocytes (Fig. 8, *lanes 2* and *4*). The endogenous mouse tyrosinase band corresponded in size to the human TYR oligomer translated in the semipermeabilized cell system using wild type melanocytes. The cross-linking efficiency of endogenous tyrosinase in wild type mouse melanocyte was low because a large fraction remained monomeric after BMH treatment (Fig. 8, compare *lane 1* with *lane 2*). This result suggested that proper folding of tyrosinase involves the formation of a number of intramolecular disulfide bonds and

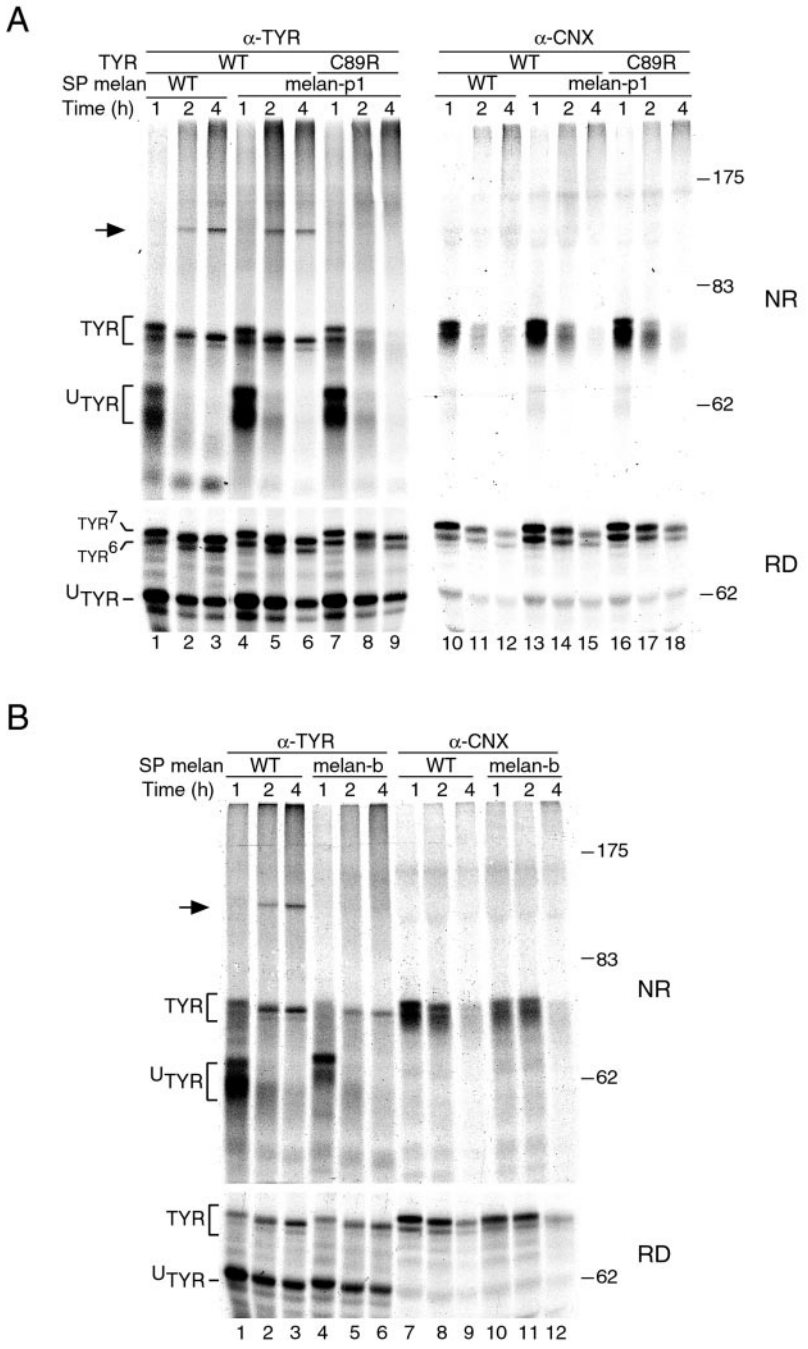

FIG. 7. **Oligomerization of TYR is impaired by mutation in TYR or Tyrp1.** WT and mutant TYR(C89R) were translated with reticulocyte lysate in the presence of semipermeabilized mouse melanocytes expressing wild type tyrosinase (WT), mutant p-protein (melan-p1; *A*, *lanes 4–9* and *13–18*) or mutant Tyrp1 (melan b; *B, lanes 4–6* and *10–12*). At the end of increasing periods of maturation, radioactively labeled TYR was subjected to immunoprecipitation with anti-TYR or anti-CNX antibodies before separation by SDS-PAGE under nonreducing (*NR*) and reducing (*RD*) conditions. An *arrow* designates TYR oligomer.

that only a small number of free thiols are available for intermolecular cross-linking. In contrast, the cross-linking efficiency for Tyr(C85S) from albino melanocytes approached 100% (Fig. 8, compare *lane 3* with *lane 4*), suggestive of multiple Cys residues accessible for intermolecular bonding by BMH on the malfolded protein. Noticeably, the Tyr(C85S) intermolecular bonding produced complexes of ≥175 kDa, not the 150-kDa conformation observed in wild type melanocytes, suggesting the involvement of different protein(s) in the complex. Altogether, these results confirmed that tyrosinase oligomerizes to a ~150-kDa conformation in cells harboring wild type but not mutant inactive tyrosinase.

DISCUSSION

We developed a semipermeabilized melanocyte system that permitted the dissection of the ER maturation process for TYR. The procedure facilitated direct and rigorous accounting of wild type and mutant protein levels because TYR was the only protein generated by the *in vitro* translation of radiolabeled proteins. Furthermore, this system did not support the anterograde transport from the ER to the Golgi, as indicated by the inability of TYR to acquire complex sugars, allowing ER maturation events to be monitored independently from downstream processes in the secretory pathway.

We provide for the first time the evidence that TYR oligomerizes as it becomes properly folded in the ER. The maturation steps resolved by the *in vitro* semipermeabilized melanocyte system included binding of nascent TYR to the lectin chaperones CNX and CRT in a monoglucosylated-dependent manner. This binding lasted until intramolecular disulfides were in place and a protease-resistant conformation was reached. Proper folding was followed by oligomerization before TYR exit from the ER. The inability of albino inactive mutant TYR(C89R) to oligomerize supports the conclusion that the

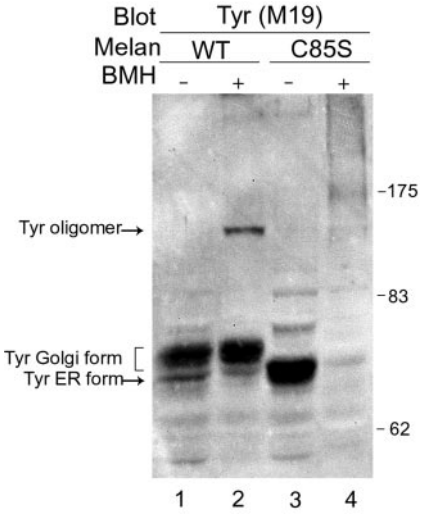

FIG. 8. **Identification of tyrosinase oligomer in mouse melanocytes.** WT and c-albino mutant (*C85S*) mouse melanocytes were treated with the thiol-reactive, homobifunctional, and membrane-permeable chemical cross-linker BMH before membrane lysis (*lanes 2* and *4*). The cross-linker was quenched with β-mercaptoethanol during lysis. Tyrosinase was visualized by Western blotting with anti-tyrosinase antibodies after separation in reducing SDS-PAGE.

normal productive pathway of TYR maturation involves oligomerization in the ER. This oligomer is likely formed by an intermolecular disulfide bond because it was disrupted under reduced conditions. Furthermore, we identified Cys^{500} in the cytoplasmic tail of TYR as the key residue for dimerization because the protein failed to oligomerize in its absence, as shown for tailless TYR and for a point mutant in which Cys^{500} was substituted by Ser (TYR(C500S)).

In eukaryotic cells, the lumen of the ER maintains an oxidizing environment, whereas the cytosol provides reducing conditions. Disulfide bond formation, in general, occurs in the ER lumen but has also been observed in the cytosol (10). Our initial detection of TYR oligomers *in vitro* was facilitated by the creation of a nonphysiological oxidizing environment outside the ER of semipermeabilized melanocytes with the oxidizing agent FAD. We postulate that under normal cellular redox conditions, TYR forms a noncovalent oligomer by the alignment of the transmembrane or cytoplasmic tail regions, bringing the juxtamembrane regions of the cytoplasmic tail of TYR (Cys^{500}) together. Therefore, exposing Cys^{500} to an oxidizing environment supported the formation of an intermolecular disulfide bond that covalently trapped the oligomer. Cross-linking studies with intact melanocytes possessing wild type but not misfold mutant tyrosinase verified that this oligomer also occurred under physiological redox conditions in cells.

The mobility of the oligomer on nonreducing SDS-PAGE as a ~150-kDa protein and results from hydrodynamic studies demonstrating a 7.5 S complex were consistent with TYR forming a homodimer within the ER. The conclusion that this was a TYR homodimer was also supported by our inability to immunoprecipitate the oligomer with antibodies against a homologous protein of similar size, Tyrp1 (data not shown), known to exist in a melanogenic complex with TYR (35). Furthermore, the cytosolic tail of Tyrp1 lacks Cys residues, and thus failure to form heterodimers with TYR is consistent with our results demonstrating a requirement for Cys^{500} in TYR for intermolecular disulfide bonding. The same argument can also be applied to another homologous protein, DOPAchrome tautomerase (DCT; previously named Tyrp2), because this protein also lacks Cys residues in its cytosolic tail. Our conclusion is in agreement with the observations that purified tyrosinase behaves as a homodimer by size-exclusion chromatography in nonionic detergent (36). However, we cannot exclude the possibility that the complex contained additional proteins before ER exit that were not stabilized under the experimental conditions used here.

TABLE I
Maturation and oligomerization of human TYR in semipermeabilized cells

TYR	Semipermeabilized cells	CNX release	Oligomers
WT	WT melanocytes	+	+
	CHO	−	−
	Tyr(C85S) melanocytes, c-albino	+	−
	Melan p-(p^{cp}/p^{25H}) melanocytes	+	+
	Melan b (Tyrp1(C86Y)) melanocytes	+	−
	Melanoma cells (WT TYR)	+	−
C89R	WT melanocytes	+	−
	Melan p-(p^{cp}/p^{25H}) melanocytes	+	−
ΔTail	WT melanocytes	+	−
C500S	WT melanocytes	+	−

Detailed analysis using several semipermeabilized cellular systems as the source for *in vitro* translation-maturation processes demonstrated requirement for melanocyte-specific factors (Table I). TYR did not mature properly using heterologous sources. The translocation of TYR into rough ER-derived microsomes from pancreatic acinar cells generated a protein that remained associated with CNX, was trypsin-sensitive, and was unable to oligomerize. Similarly, release from CNX and oligomerization were not observed when CHO cells were used as the source for semipermeabilized cells for TYR maturation. The persistent binding to CNX indicated that TYR remained misfolded in these heterologous cellular systems because it was continually reglucosylated by UDP-glucose:glycoprotein glucosyltransferase, an enzyme that recognizes non-native structures (18).

According to the results described here, two main functions were required for TYR oligomerization, *i.e.* its own enzymatic activity and Tyrp1 (Table I). We ruled out the involvement of the p-protein. In p-melanocytes with null mutations in the *pink-eyed dilution* gene, tyrosinase is mislocalized to the extracellular compartment, and a fraction is retained in the ER (34, 37). However, in our hands, CNX binding, glycan trimming, and oligomerization of TYR produced in semipermeabilized melan-p melanocytes were indistinguishable from those produced in semipermeabilized wild type melanocytes.

Several studies demonstrated that TYR is stabilized by its own enzymatic activity (29, 38). Stimulation of TYR in intact human melanoma cells by DOPA and tyrosine enhanced TYR maturation in the ER and its transport to the Golgi (29). Likewise, co-expression of wild type protein with temperature-sensitive TYR mutants corrected the mutant conformation defect in an activity-dependent manner (38). The results from the *in vitro* translation-maturation system described here are consistent with these observations because WT TYR failed to oligomerize when allowed to mature in the semipermeabilized c-albino melanocytes with null Tyr(C85S) mutant protein and in melanoma cells with extremely low TYR activity.

In vivo, tyrosinase degradation is enhanced in mouse melan-b melanocytes defective for Tyrp1 (32). Here, a role for Tyrp1 in the proper maturation of TYR within the ER was demonstrated by the failure of TYR to dimerize in the ER of semipermeabilized melan-b melanocytes after its proper maturation and release from CNX. Tyrp1 is the most abundant of the melanocyte-specific proteins, but its activity remains controversial. Immunoprecipitated human Tyrp1 was shown to possess catalase activity, but the studies did not rule out that the activity was derived from an associated protein (39). In

murine melanocytes, Tyrp1 was reported to function as a 5,6-dihydroxyindole-2-carboxylic acid oxidase, the downstream substrate produced by the tyrosinase reaction (40). However, the same function could not be demonstrated for human Tyrp1 by some of the same investigators (41). Furthermore, another group demonstrated that ectopic mouse Tyrp1 expressed in transfected fibroblasts possessed DOPAchrome tautomerase activity (42). Therefore, at this point, it remains to be determined how Tyrp1 supports TYR oligomerization.

The significance of TYR oligomerization in *in vivo* maturation processes and transport to the Golgi is not yet clear. Mouse tyrosinase is in large part normally processed in melan-b melanocytes, and the cells (as well as the mutant mouse) suffer only minor reduction in pigmentation (brown instead of black). In addition, TYR can induce pigmentation in nonmelanocytic cells (43). However, the overall efficiency of pigmentation in nonmelanocytic cells expressing tyrosinase is much lower than that in melanocytes. This is due not only to the targeting of TYR to lysosomes in the absence of melanosomes, the subcellular organelles for melanin formation, but also to sluggish maturation, as demonstrated in parallel experiments using melanocytes and CHO cells (38). Perhaps oligomerization is not obligatory for TYR activity or exit from the ER but enhances this process.

Time course analysis showed that TYR dimerization occurred after TYR was released from CNX. This sequence of events is consistent with previous results obtained with the viral glycoprotein hemagglutinin, where its folding and oligomerization occurred after release from chaperones (44). In addition, CNX/CRT binding was not obligatory for TYR oligomerization because dimers were observed after inhibition of glucose trimming. However, the overall efficiency of oligomerization was greatly decreased under these circumstances, suggestive of a role for the lectin chaperones in enhancing the fidelity of the maturation process. The inhibition of glucose trimming and subsequent CNX/CRT binding in mouse melanoma cells by glucosidase inhibition has been found previously to greatly decrease melanin production (16). In that report, inactive tyrosinase was transported to melanosomes. Evidently, CNX/CRT binding to tyrosinase aided in the efficient folding of the protein and served the quality control function of retaining non-native protein in the ER. Abolishing the interaction not only created inactive protein but also disrupted its retention by the quality control machinery of the ER.

CNX binding is mediated by monoglucosylated glycans. Glycoproteins can persist in the monoglucosylated state through repeated deglucosylation and reglucosylation cycles. UDP-glucose:glycoprotein glucosyltransferase reglucosylates proteins containing misfolded or immature structures (18). The general notion is that reglucosylation and subsequent CNX binding persist until a protein is correctly folded or assembled. Under certain conditions in our system, TYR binding to CNX ceased indicating proper folding, but the protein did not oligomerize. This pattern was observed with the folding-defective TYR(C89R), with TYR mutants lacking the critical residue required for oligomerization (C500S and ΔTail) expressed in semipermeabilized wild type or melan-p1 melanocytes, and with wild type protein expressed in semipermeabilized albino and Tyrp1-defective mouse melanocytes (Table I). How could a defective protein be released from CNX and remain stable within the ER?

Defective proteins are generally retained in the ER and subsequently targeted for degradation by the proteasome through the ER protein-associated degradation pathway (45). EDEM, a mammalian ER type II membrane glycoprotein, has recently been implicated as a quality control receptor that extracts proteins from the CNX binding cycle and sorts them for destruction (46, 47). Like CNX, EDEM is also a lectin, but its binding is mediated by the slow trimming of a mannose residue by ER mannosidase I to the mannose 8 form. Therefore, the release of the malfolded TYR(C89R) from CNX was likely due to its accumulation on EDEM. Generally, binding to EDEM is associated with substrate dislocation to the cytosol and rapid proteasomal degradation. However, to optimize translation, the *in vitro* system used in our studies contains hemin, a suppressor of an inhibitor of the initiation factor eIF2α. Hemin also inhibits the proteasome, thus allowing TYR(C89R) stabilization within the ER.

The use of reduced biological systems that support mechanistic studies can help in elucidating the details of how the cellular machinery operates. Through the use of semipermeabilized melanocytes, we have been able to draw a detailed picture of the ER maturation steps for TYR. Future studies should explain how active tyrosinase and Tyrp1 help with the maturation of TYR. In addition, this *in vitro* system will permit examination of the quality control components participating in ER retention and sorting of defective proteins to the ER protein-associated degradation pathway reported to operate in albino melanocytes and melanoma cells.

Acknowledgments—We thank Drs. Vince Hearing and D. C. Bennett for the melan-b and melan-p1 melanocytes and Donna LaCivita for maintaining the cells in culture.

REFERENCES

1. Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H. (1949) *J. Biol. Chem.* **178,** 185–195
2. Marks, M. S., and Seabra, M. C. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 1–11
3. Oetting, W. S., and King, R. A. (1999) *Hum. Mutat.* **13,** 99–115
4. Halaban, R., Svedine, S., Cheng, E., Smicun, Y., Aron, R., and Hebert, D. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5889–5894
5. Berson, J. F., Frank, D. W., Calvo, P. A., Bieler, B. M., and Marks, M. S. (2000) *J. Biol. Chem.* **275,** 12281–12289
6. Toyofuku, K., Wada, I., Spritz, R. A., and Hearing, V. J. (2001) *Biochem. J.* **355,** 259–69
7. Halaban, R., Chang, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V., and Hebert, D. N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6210–6215
8. Halaban, R., Patton, R. S., Cheng, E., Svedine, S., Trombetta, E. S., Wahl, M. L., Ariyan, S., and Hebert, D. N. (2002) *J. Biol. Chem.* **277,** 14821–14828
9. Schnell, D. J., and Hebert, D. N. (2003) *Cell* **112,** 491–505
10. Sevier, C. S., and Kaiser, C. A. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 836–847
11. Daniels, R., Kurowski, B., Johnson, A. E., and Hebert, D. N. (2003) *Mol. Cell* **11,** 79–90
12. Újvári, A., Aron, R., Eisenhaure, T., Cheng, E., Parag, H. A., Smicun, Y., Halaban, R., and Hebert, D. N. (2001) *J. Biol. Chem.* **276,** 5924–5931
13. Hammond, C., Braakman, I., and Helenius, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 913–917
14. Hebert, D. N., Foellmer, B., and Helenius, A. (1995) *Cell* **81,** 425–433
15. Takahashi, H., and Parsons, P. G. (1992) *J. Invest. Dermatol.* **98,** 481–487
16. Petrescu, S. M., Petrescu, A.-J., Titu, H. N., Dwek, R. A., and Platt, F. M. (1997) *J. Biol. Chem.* **272,** 15796–15803
17. Branza-Nichita, N., Petrescu, A. J., Dwek, R. A., Wormald, M. R., Platt, F. M., and Petrescu, S. M. (1999) *Biochem. Biophys. Res. Commun.* **261,** 720–725
18. Sousa, M., and Parodi, A. J. (1995) *EMBO J.* **14,** 4196–4203
19. Helenius, A. (1994) *Mol. Biol. Cell* **5,** 253–265
20. Svidersjaya, E. V., Bennett, D. C., Ho, L., Lee, S. T., and Spritz, R. A. (1997) *J. Invest. Dermatol.* **108,** 30–34
21. Zdarsky, E., Favor, J., and Jackson, I. J. (1990) *Genetics* **126,** 443–449
22. Jackson, I. J., Chambers, D., Rinchik, E. M., and Bennett, D. C. (1990) *Genetics* **126,** 451–459
23. Braakman, I., Hoover-Litty, H., Wagner, K. R., and Helenius, A. (1991) *J. Cell Biol.* **114,** 401–411
24. Wilson, R., Allen, A. J., Oliver, J., Brookman, J. L., High, S., and Bulleid, N. J. (1995) *Biochem. J.* **307,** 679–687
25. Cannon, K. S., Hebert, D. N., and Helenius, A. (1996) *J. Biol. Chem.* **271,** 14280–14284
26. Wilson, C. M., Farmery, M. R., and Bulleid, N. J. (2000) *J. Biol. Chem.* **275,** 21224–21232
27. Chen, W., Helenius, J., Braakman, I., and Helenius, A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6229–6233
28. Tatu, U., Hammond, C., and Helenius, A. (1995) *EMBO J.* **14,** 1340–1348
29. Halaban, R., Cheng, E., Svedine, S., Aron, R., and Hebert, D. N. (2001) *J. Biol. Chem.* **276,** 1193–11938
30. Oetting, W. S. (1998) in *The Pigmentary System* (Nordlund, J. J., Boissy, R., Hearing, V. J., King, R. A., and Ortonne, J.-P., eds) 1st Ed., pp. 231–249, Oxford University Press, New York
31. Potterf, S. B., and Hearing, V. J. (1998) *Biochem. Biophys. Res. Commun.* **248,** 795–800
32. Kobayashi, T., Imokawa, G., Bennett, D. C., and Hearing, V. J. (1998) *J. Biol. Chem.* **273,** 31801–31805

33. Puri, N., Gardner, J. M., and Brilliant, M. H. (2000) *J. Invest. Dermatol.* **115,** 607–613
34. Chen, K., Manga, P., and Orlow, S. J. (2002) *Mol. Biol. Cell* **13,** 1953–1964
35. Orlow, S. J., Zhou, B. K., Chakraborty, A. K., Drucker, M., Pifko-Hirst, S., and Pawelek, J. M. (1994) *J. Invest. Dermatol.* **103,** 196–201
36. Jimenez-Cervantes, C., Martinez-Esparza, M., Solano, F., Lozano, J. A., and Garcia-Borron, J. C. (1998) *Biochem. Biophys. Res. Commun.* **253,** 761–767
37. Toyofuku, K., Valencia, J. C., Kushimoto, T., Costin, G., Virador, V. M., Vieira, W. D., Ferrans, V. J., and Hearing, V. J. (2002) *Pigment Cell Res.* **15,** 217–224
38. Halaban, R., Cheng, E., and Hebert, D. N. (2002) *J. Invest. Dermatol.* **119,** 481–488
39. Halaban, R., and Moellmann, G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4809–4813
40. Kobayashi, T., Urade, K., Winder, A., Jimenez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., Garcia-Borron, J. C., and Hearing, V. J. (1994) *EMBO J.* **13,** 5818–5825
41. Boissy, R. E., Sakai, C., Zhao, H., Kobayashi, T., and Hearing, V. J. (1998) *Exp. Dermatol.* **7,** 198–204
42. Winder, A. J., Wittbjer, A., Rosengren, E., and Rorsman, H. (1998) *J. Cell Sci.* **106,** 153–166
43. Bouchard, B., Fuller, B. B., Vijayasaradhi, S., and Houghton, A. N. (1989) *J. Exp. Med.* **169,** 2029–2042
44. Hebert, D. N., Foellmer, B., and Helenius, A. (1996) *EMBO J.* **15,** 2961–2968
45. Bonifacino, J. S., and Weissman, A. M. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 19–57
46. Molinari, M., Calanca, V., Galli, C., Lucca, P., and Pagnetti, P. (2003) *Science* **299,** 1397–1400
47. Oda, Y., Hosokawa, N., Wada, I., and Nagata, K. (2003) *Science* **299,** 1394–1397