Carbohydrates act as sorting determinants in ER-associated degradation of tyrosinase

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Summary

The endoplasmic reticulum (ER) quality-control machinery maintains the fidelity of the maturation process by sorting aberrant proteins for ER-associated protein degradation (ERAD), a process requiring retro-translocation from the ER lumen to the cytosol and degradation by the proteasome. Here, we assessed the role of N-linked glycans in ERAD by monitoring the degradation of wild-type (Tyr) and albino mutant (Tyr(C85S)) tyrosinase. Initially, mutant tyrosinase was established as a genuine ERAD substrate using intact melanocyte and semi-permeabilized cell systems. Inhibiting mannose trimming or accumulating Tyr(C85S) in a monoglucosylated form led to its stabilization, supporting a role for lectin chaperones in ER retention and proteasomal degradation. In contrast, ablating the lectin chaperone interactions by preventing glucose trimming caused a rapid disappearance of tyrosinase, initially due to the formation of protein aggregates, which were subsequently degraded by the proteasome. The co-localization of aggregated tyrosinase with protein disulfide isomerase and BiP, but not calnexin, supports an ER organization, which aids in protein maturation and degradation. Based on these studies, we propose a model of tyrosinase degradation in which interactions between N-linked glycans and lectin chaperones help to minimize tyrosinase aggregation and also target non-native substrates for retro-translocation and subsequent degradation.

Key words: N-linked glycans, Quality control, Molecular chaperones, Protein aggregation

Introduction

The protein maturation process is monitored in the endoplasmic reticulum (ER) by stringent quality control machinery that sorts misfolded proteins for ER-associated protein degradation (ERAD). This is a complex process involving recognition, ER retention, and retro-translocation of proteins into the cytosol followed by deglycosylation, ubiquitination, and subsequent proteolysis by the proteasome (Bonifacino and Weissman, 1998; Plemper and Wolf, 1999; Ellgaard and Helenius, 2003). Disruption of protein maturation within the ER is increasingly associated with a number of human diseases including cystic fibrosis, lung and liver diseases, and the genetic disorder albinism (Halaban et al., 2000; Rutishauser and Speiss, 2002; Thomas et al., 1995). As a consequence, it is essential to characterize and understand the sorting signals recognized by ER quality-control components.

N-linked glycans are large, flexible and hydrophilic moieties that are modified by cellular glycosidases and transferases (Rudd et al., 2001). In recent years, it has emerged that these modifications serve as tags on maturing proteins for the recruitment of quality-control components (Helenius and Aebi, 2001). During the co-translational translocation of a polypeptide into the ER, the fourteen-member carbohydrate ($Glc_3Man_9GlcNAc_2$) is transferred to the asparagine of an N-linked glycosylation consensus sequence. Shortly thereafter, glucosidases I and II rapidly remove the first two glucose residues. The resulting monoglucosylated glycan can then be recognized by the ER-resident lectin chaperone calnexin (CNX) and its soluble paralogue, calreticulin (CRT) (Hammond et al., 1994; Hebert et al., 1995; Ou et al., 1993). The removal of the final glucose residue by glucosidase II prevents further binding by these lectin chaperones. In the event of the glycoprotein not acquiring its native conformation, the folding sensor UDP-glucose: glycoprotein glucosyltransferase (GT) can reglucosylate the glycan, permitting another round of lectin chaperone binding (Sousa and Parodi, 1995). Continuous rounds of chaperone binding and release due to reglucosylation by GT and subsequent deglucosylation by glucosidase II can keep a glycoprotein in the ER until its folding is complete. Proteins that do not reach their native state are believed to exit the binding cycle through the action of ER mannosidase I, which generates the Man8 glycoform (Frenkel et al., 2003; Herscovics et al., 2002; Jakob et al., 1998; Liu et al., 1999).

Recent studies have linked the trimming of mannose and glucose residues to the targeting of glycoproteins for degradation (for reviews see Daniels et al., 2004; Helenius and Aebi, 2001; Lehrman, 2001; Spiro, 2000). Glycoproteins that are otherwise rapidly degraded become stable when expressed in mannosidase-deficient cells, or in cells treated with mannosidase inhibitors, suggesting that mannose trimming is a prerequisite for sorting of glycoproteins to the proteasome

(Jakob et al., 1998; Liu et al., 1999; Wang and Androlewicz, 2000; Yang et al., 1998). This led to the discovery of a putative mannose-specific lectin receptor in yeast (termed Htm1p or Mnl1p) and mammals (termed EDEM) (Hosokawa et al., 2001; Jakob et al., 2001; Molinari et al., 2003; Nakatsukasa et al., 2001; Oda et al., 2003). In contrast, the role of glucose trimming and lectin chaperone interaction in ERAD is less clear. In some studies, the inhibition of glucose trimming results in enhanced degradation (Cabral et al., 2000; Moore and Spiro, 1993; Wilson et al., 2000; Yang et al., 1998), whereas in others glycoproteins are either stabilized (Marcus and Perlmutter, 2000; Oda et al., 2003), or unaffected (Ayalon-Soffer et al., 1999; Fagioli and Sitia, 2001; Moussali et al., 1999). Furthermore, ablating lectin chaperone interactions by preventing glucose removal is associated with degradation through a non-proteasomal pathway (Cabral et al., 2000).

We have investigated the role of glucose and mannose trimming in the proper maturation and degradation of glycoproteins by monitoring the fate of tyrosinase. Tyrosinase is a melanocyte-specific type-I membrane glycoprotein responsible for converting tyrosine to DOPA and DOPAquinone, the first intermediates in melanin synthesis (Lerner et al., 1949). It is a useful model substrate for the study of ER-associated glycoprotein degradation. Its enzymatic activity can be easily monitored by observing changes in cell pigmentation. Tyrosinase peptides have also been characterized as a classical substrate of the MHC Class-I antigen presentation pathway (Mosse et al., 1998). Mutations in tyrosinase are the cause of oculocutaneous albinism type 1 (OCA1) (Oetting and King, 1999). In albino melanocytes the quality-control machinery recognizes the inactive tyrosinase mutants as misfolded and retains the protein in the ER (Berson et al., 2000; Halaban et al., 2000; Toyofuku et al., 2001). Our results indicate that the prevention of mannose trimming or accumulation of tyrosinase in a monoglucosylated form lead to its stabilization, while the inhibition of glucose trimming causes transient aggregation. Eventually, the aggregates are dissociated and targeted for degradation by the proteasome. Therefore, the status of the glycans on tyrosinase is used by the cell as a maturation and quality-control tag to sort glycoproteins within the early secretory pathway.

Materials and Methods

Cell lines and reagents

Immortalized mouse melanocytes established from a wild-type black B10BR mouse served as wild-type controls (Tamura et al., 1987). Albino melan c melanocytes homozygous for the *c*-locus (*Tyr*) C85S mutation termed Tyr(C85S) were the source of albino mutant tyrosinase (Bennett et al., 1989).

Easy Tag [^{35}S]-Methionine/Cysteine and Zysorbin were from Perkin-Elmer Life Sciences (Beverly, MA) and Zymed Laboratories (San Francisco, CA), respectively. The transcription kits mMessage mMachine and T7 were from Ambion (Austin, TX). Rabbit Reticulocyte Lysate (RRL), Untreated RRL (URL), and RNasin were from Promega Corp. (Madison, WI). Lactacystin (LCT), clasto-lactacystin β-lactone (β-lac), tunicamycin, MG132 were from Calbiochem (San Diego, CA). All other reagents were from Sigma-Aldrich (St Louis, MO).

Immunoblotting

Cells were lysed in 2% CHAPS in 90 mM Hepes and 50 mM NaCl (pH 7.5) termed HBS. Total protein was determined using the BioRAD protein assay (Bio-Rad Laboratories, Hercules, CA). Tyrosinase was immunoblotted with Pep7 (V. Hearing, NIH, Bethesda, MD) or M-19 (goat, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. Proteasomes were detected using anti-proteasome antibodies against the α-subunits (Zymed Laboratories, Inc., San Francisco, CA). Antigen-antibody complexes were visualized by ECL (Amersham Pharmacia Biotech., Piscataway, NJ).

Subcellular fractionation, metabolic labeling and immunoprecipitations

Melanocytes were detached from the culture flask with 0.25% trypsin solution in MEMS and centrifuged at 6000 ***g*** for 5 minutes. Following a 2-hour starvation in Met/Cys free-RPMI 1640 medium supplemented with dialyzed calf serum (3%), glutamine (1%), and TPA (50 ng/ml), melanocytes were radiolabeled with [^{35}S]Met/Cys (0.7 mCi/ml) for 30 minutes (pulse), the radioactive medium was removed and the cells were further incubated in regular medium (chase) for increasing periods of time. When indicated, melanocytes were incubated in medium supplemented with 25 μM LCT, 1 mM deoxymannojrimycin (DMJ), or 500 μM *n*-butyl deoxynojirimycin (DNJ) during the starvation, pulse, and chase periods. The harvested cells were suspended in PBS supplemented with 20 mM N-ethylmaleimide (NEM). Cell pellets were lysed in 2% CHAPS HBS buffer. Extracts were immunoprecipitated with polyclonal anti-tyrosinase antibodies and immune-complexes were then resolved by SDS-PAGE and quantified by phosporimaging (Halaban et al., 1997). Cytosol was separated from cell membranes as previously described (Wiertz et al., 1996).

Construction of plasmids

The plasmid pSP72/K^{b}SS-TYR was constructed as described (Újvári et al., 2001). To create TYR(C89R), the human orthologue of Tyr(C85S), pSP72/K^{b}SS-TYR was subjected to site-directed mutagenesis in which the cysteine at position 89 was changed to arginine using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Transcription, translation and translocation

Messenger RNA was prepared by in vitro run-off transcription of linearized cDNA (Újvári et al., 2001). Radioactive ^{35}S-labeled tyrosinase was translated for 1 hour in RRL in the presence of canine pancreas microsomes (MS) or semi-permeabilized (SP) melanocytes (Francis et al., 2003; Hebert et al., 1995). The standard translation mixture consisted of 1.75 μl MS or 3×10^{5} semi-permeabilized cells, 1 μl RNasin, 13 μl RRL, 0.5 μl of 1 mM amino acid mixture minus methionine and cysteine, [^{35}S]Met/Cys (0.63 μCi), 3.2 mM DTT, 87 mM KCl, 2 mM oxidized glutathione (GSSG), and 500 ng mRNA. Where indicated, the standard translation mixture was treated with 1 mM DMJ or DNJ for 10 minutes at 27°C prior to translation. SP-melanocytes were prepared from subconfluent melanocytes permeabilized with 20 μg/ml digitonin (Francis et al., 2003; Wilson et al., 1995).

SP-melanocyte degradation assay

Protein synthesis was prevented after translation in RRL in the presence of SP-melanocytes by the addition of 1 mM cycloheximide (CHX). SP-melanocytes were harvested by centrifugation at 12,000 ***g*** for 2 minutes and were resuspended in KHM buffer (110 mM KOAc, 2 mM MgOAc, 20 mM HEPES, pH 7.2). After repeated centrifugation to remove any remaining cytosol, SP-melanocytes were resuspended in KHM buffer, divided into aliquots of 40 μl, and collected by centrifugation at 12,000 ***g*** for 2 min. They were resuspended in 5 μl

URL or 5 μl KHM buffer in the presence of an Energy Regeneration System (ERS, 0.5 mM ATP, 1.6 mg/ml creatine phosphokinase, 10 mM phosphocreatine, 5 mM $MgCl_2$, 40 mM Tris-HCl, and 2 mM DTT). When designated, 1 mM DNJ and DMJ, or 300 μM clasto-lactacystin β-lactone was added to the SP-melanocyte degradation system. For ATP depletion studies, SP-melanocytes and URL were pretreated with 0.1 units/ml hexokinase, 0.22 M glucose, and 6.5 mM $MgCl_2$ for 1 hour at 37°C before incubation with the ERS minus ATP. Degradation was terminated by the addition of NEM and cooling the samples on ice.

Trichloroacetic acid precipitation degradation assay

The level of intact versus degraded tyrosinase generated in the presence of SP-melanocytes was assessed by measuring the total radioactive protein as a function of radioactivity in the TCA-soluble material. Briefly, human albino TYR(C89R) cDNA was translated in RRL in the presence of SP-melanocytes, which had been preincubated with inhibitors. Following centrifugation at 140,000 ***g*** for 10 minutes to remove untranslocated protein, membranes were resuspended in URL and an ATP-regenerating system. Aliquots of this mixture were then precipitated with 20% TCA on ice for 30 minutes and centrifuged at 16,000 ***g*** for 15 minutes. The supernatants were then counted in ten volumes of scintillation fluid in a Beckman LS6500 scintillation counter. The percentage remaining was calculated using $100–[(C_n–C_0)/(T–C_0)]$, where C_n=soluble counts at each time point, C_0=soluble counts at T=0, and T=total counts (both supernatant and pellet) per aliquot (Oberdorf and Skach, 2002).

Indirect immunofluorescence

Melanocytes were incubated in medium supplemented with DNJ (0-1 mM) for 5-8 hours, fixed in 4% formaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS, and then incubated with antibodies (diluted in 0.1% BSA/PBS) against protein disulfide isomerase (PDI) (SPA-890), BiP (SPA-826), calnexin (SPA-860), each from Stressgen Biotechnology Inc. (Victoria, Canada), and tyrosinase (M-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Primary antibodies were detected with rhodamine- or fluorescein-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Indirect immunofluorescence was visualized with an inverted Bio-Rad MRC-600 Laser Confocal Microscope.

Results

Tyrosinase is an ERAD substrate

A radioactive pulse-chase experiment was used to assess the stability of Tyr in wild-type and albino mouse melanocytes. At the end of the 30-minute pulse, Tyr migrated as a 70-kDa protein, characteristic of a high-mannose ER species (Fig. 1A, Tyr, arrowhead) (Halaban et al., 1997; Halaban et al., 2000). After 1 hour of chase, the 80-kDa mature isoform of Tyr began to accumulate indicating the transfer of complex carbohydrates in the Golgi (arrow). In contrast, the albino ER-retained Tyr(C85S) mutant persisted as a 70-kDa protein doublet even after 8 hours of chase (Fig. 1A, Tyr(C85S)) (Halaban et al., 2000). The 70-kDa Tyr doublet observed in the wild-type and albino melanocytes resulted from inefficient glycosylation of the six-consensus glycosylation sites in mouse Tyr [(Újvári et al., 2001) and data not shown]. Determination of both mature and immature tyrosinase levels revealed that Tyr(C85S) was less stable than wild-type Tyr, exhibiting half-lives of 3 and 6 hours, respectively (Fig. 1A, plot).

Previous studies show that wild-type tyrosinase in human amelanotic melanoma cells is degraded by the proteasome

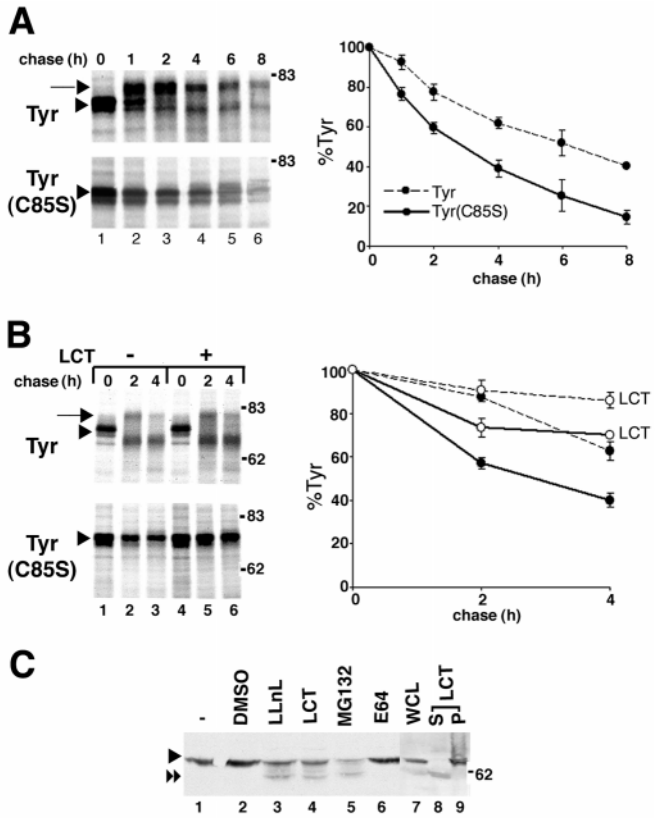

Fig. 1. Tyr and Tyr(C85S) are proteasome substrates. (A) Wild-type Tyr or mutant Tyr(C85S) mouse melanocytes were pulsed with [^{35}S]-Met/Cys for 30 minutes and chased for the indicated times. Lysates were immunoprecipitated with anti-tyrosinase antibodies. Arrowheads and arrows indicate high-mannose and complexed forms of tyrosinase, respectively. The plot displays the quantification of five separate experiments. (B) Tyr(C85S) melanocytes were treated as in A, except half of the samples were incubated with 25 μM lactacystin (LCT) during the starvation, pulse, and chase periods. The plot displays the quantification of four separate experiments. (C) Tyr(C85S) melanocytes were incubated for 4 hours in the presence of various inhibitors: DMSO, 50 μM Leu-Leu nor-Leucinal (LLnL), 25 μM LCT, 50 μM MG132, or 50 μM E64 as indicated. Cell lysates were then either directly subjected to immunoblotting using anti-tyrosinase antibodies, or fractured and ultracentrifuged to separate the cytosol (S) from the membrane (P). Unglycosylated tyrosinase is indicated by a double arrowhead.

(Halaban et al., 1997). To determine if murine tyrosinase was degraded by the proteasome, pulse-chase analysis was performed using pigmented and albino melanocytes in the absence or presence of the specific proteasome inhibitor, lactacystin (LCT) (Fig. 1B) (Lee and Goldberg, 1998). LCT stabilized both the wild-type and mutant Tyr proteins but displayed a more pronounced effect for the mutant form. After a 4-hour chase, the levels of Tyr(C85S) increased approximately two-fold from 38% to 73%, and wild-type levels from 66% to 89% in response to LCT indicating that murine tyrosinase is degraded by the proteasome.

In order for murine tyrosinase to be a substrate for the proteasome it must first be retro-translocated from the ER lumen to the cytosol where the protein is deglycosylated and ubiquitinated prior to degradation. Since deglycosylated

tyrosinase was not observed in the radioactive pulse-chase experiments examining murine Tyr and Tyr(C85S), we tested if deglycosylated tyrosinase could be observed under steady-state conditions after proteasome inhibition. Albino Tyr(C85S) melanocytes were incubated with a cysteine protease inhibitor (LLnL), specific proteasome inhibitors (MG132 and LCT), or a lysosomal cysteine protease inhibitor (E64). Deglycosylated tyrosinase (60 kDa) accumulated only in the lysates from cells treated with proteasome inhibitors (Fig. 1C, lanes 3-5, double arrowhead). The deglycosylated nature of the 60-kDa protein was confirmed by its inability to bind concanavalin A and the absence of any mobility shift after PNGaseF treatment (data not shown).

To determine if the 60-kDa protein corresponded to a soluble cytosolic form of tyrosinase, the cytosol of LCT-treated albino melanocytes was separated from the membrane fraction by the ultracentrifugation of fractured cells. The 60-kDa deglycosylated Tyr(C85S) localized to the cytosol, whereas the 70-kDa protein co-localized with the membrane fraction (Fig. 1C, lanes 8 and 9). Together, the results show that both the wild-type and albino tyrosinase are genuine ERAD substrates and albino tyrosinase is readily retro-translocated from the ER lumen to the cytosol where it undergoes deglycosylation prior to proteasomal degradation.

Tyrosinase ERAD can be reconstituted in a semi-permeabilized melanocyte system

A semi-permeabilized (SP) cell system was used to separate ER-localized events from those occurring in the cytosol and to provide a method for protein quantification that was not dependent upon inefficient immunoprecipitation (Hebert et al., 1995; McCracken and Brodsky, 1996; Wilson et al., 1995). At low concentrations, the detergent digitonin selectively permeabilizes the plasma membrane while leaving organellar membranes intact (Katz and Wals, 1985). As a consequence, cytosolic components including proteasomes can be washed away and subsequently added back in a controlled manner. Here, SP-membranes were isolated by centrifugation and resuspended in RRL optimized for the proper maturation of tyrosinase (Francis et al., 2003). Tyrosinase mRNA was then added to the mixture to translate ^{35}S-labeled tyrosinase selectively. Since only tyrosinase is labeled, an immunoprecipitation step is not required enabling the rigorous elucidation of protein levels. After translation of tyrosinase in the hemin-containing reticulocyte lysate, the tyrosinase-containing membranes were isolated by centrifugation and resuspended in RRL lacking hemin (URL, untreated RRL), which supports degradation (Xiong et al., 1999). Alternatively, membranes were resuspended in a buffer devoid of COPII and other cytosolic components preventing ER to Golgi trafficking (Beckers et al., 1987).

To reconstitute the tyrosinase degradation process, human wild-type (TYR) and albino TYR(C89R) tyrosinase that correspond to the murine wild-type (Tyr) and mutant Tyr(C85S) proteins, were translated in the presence of canine pancreatic rough ER-derived microsomes, or SP wild-type or albino mouse melanocytes. The homology and identity between human and mouse tyrosinase is 87% and 78%, respectively. As previously demonstrated (Újvári et al., 2001), two protein bands were generated in the presence of microsomes: a 60-kDa band corresponding to untranslocated and unglycosylated (^{U}TYR) protein, and a 70-kDa band representing a translocated protein which had received N-linked glycans (TYR) identical to that observed in intact melanocytes (Fig. 2A). Sixty percent of translocated TYR and TYR(C89R) was degraded after 3 hours of chase in microsomes (Fig. 2A, lanes 2 and 8), indicating that the URL supported degradation. Regardless of whether wild-type (Fig. 2A, lanes 1-6) or albino mutant (Fig. 2A, lanes 7-12) melanocytes were the source of SP cells, both TYR and TYR(C89R) were found to undergo degradation, and the mutant protein was degraded at a higher level than the wild-type protein. Thus, the level of tyrosinase degradation in SP-melanocytes closely paralleled that observed in intact wild-type and albino melanocytes.

In intact melanocytes, tyrosinase is a substrate of the proteasome, and accumulates as a deglycosylated cytosolic protein in the presence of proteasome inhibitors (Fig. 1C, lane 8). We therefore examined if human albino mutant TYR(C89R) is degraded by the proteasome in an ATP-dependent manner in albino SP-melanocytes (Fig. 2B). In the presence of cytosol and ATP, 28% TYR(C89R) was detected after 4 hours of chase. In contrast, 52% TYR was detected at this time point when ATP was depleted from the cytosol. In addition, TYR(C89R) was further stabilized in the absence of cytosol as indicated by an increase to approximately 75% when the chase was performed in buffer (Fig. 2B, compare lanes 1-3 for URL and buffer). Proteasomes detected in whole cell lysates (WCL) and SP-melanocytes suspended in URL, were absent when SP-melanocytes were suspended in buffer (Fig. 2C).

To determine if tyrosinase was degraded by the URL proteasome, TYR(C89R) was translated in the presence of SP-melanocytes and clasto-lactacystin β lactone (β-lac), a membrane-impermeable derivative of LCT (Dick et al., 1996). After a 4-hour chase in the presence of β-lac, 62% TYR(C89R) remained compared to 28% in untreated SP-cells (Fig. 2B, lanes 3 and 9; URL). These results correlate with a 73% stabilization of Tyr(C85S) in live melanocytes treated with LCT. Similar results were observed for wild-type TYR (data not shown). The requirements for cytosol, ATP, and the proteasome for efficient degradation of TYR(C89R), supported the conclusion that proteasomal degradation of TYR(C89R) was reconstituted in SP-melanocytes suspended in URL. Therefore, we have established systems to monitor the ER-associated protein degradation process using intact cells, as well as the SP-cell system, which is more amenable to experimental manipulation and quantification.

Mannose and glucose trimming are involved in sorting Tyr(C85S) to the proteasome

The stability of a variety of Tyr(C85S) glycoforms was determined to characterize the role of carbohydrate trimming in the sorting of tyrosinase for degradation. Analogues of mannose (deoxymannojirimycin; DMJ) and glucose (*n*-butyl deoxynanojirimycin, DNJ) that act as inhibitors of mannosidases and glucosidases, respectively, were employed to accumulate tyrosinase at distinct trimming steps (Fig. 3A) (Elbein, 1991; Wang and Androlewicz, 2000). The effectiveness of each inhibitor was verified by observing band-

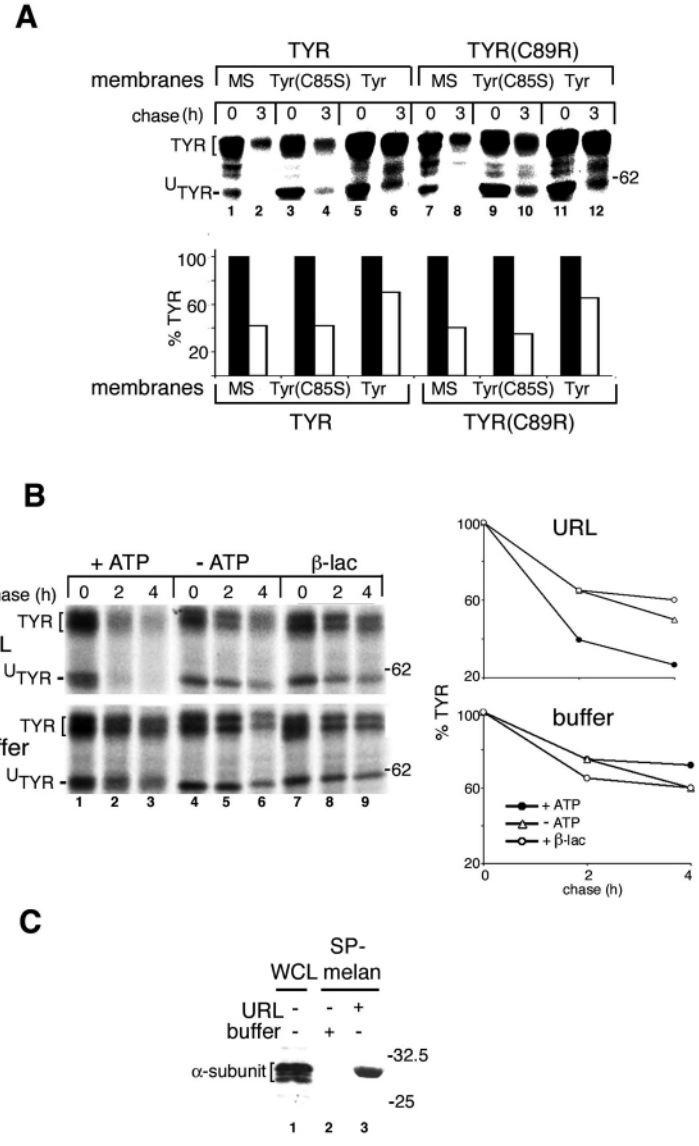

Fig. 2. Degradation of albino TYR(C89R) can be reconstituted with semi-permeabilized melanocytes. (A) TYR or TYR(C89R) mRNAs were translated in the presence of Tyr(C85S) or wild-type Tyr SP-melanocytes (membranes), or microsomes (MS). Protein synthesis was terminated by the addition of cycloheximide and MS or SP-melanocytes were isolated. Isolated membranes were resuspended in an Energy Regeneration System (ERS) in Untreated rabbit Reticulocyte Lysate (URL), and incubated at 37°C for the indicated time. TYR and ^{U}TYR indicate translocated and untranslocated tyrosinase, respectively. (B) TYR(C89R) mRNA was translated in RRL in the presence of Tyr(C85S) SP cells and either directly incubated with either URL or KHM buffer devoid of proteasomes. All the samples were incubated at 37°C for the indicated time and treated as above. (C) Proteins from Tyr(C85S) cell lysates (lane 1) or SP-melanocytes incubated in the presence of buffer (lane 2) or URL (lane 3) were separated by SDS-PAGE, and subjected to immunoblotting with antibodies to the α-subunit of the proteasome.

shifts in electrophoretic mobility. The prevention of the trimming of mannoses by DMJ resulted in the stabilization of mutant tyrosinase from 42% to 68% after a 3-hour chase (Fig. 3B, lanes 2 and 4). In contrast, the triglucosylated form of tyrosinase produced in the presence of DNJ was rapidly degraded (Fig. 3B, lane 6).

The role of monoglucosylated oligosaccharides on the stability of Tyr(C85S) was assessed in melanocytes treated with DNJ during the chase period (up to 3 hours). Since the first two glucoses are removed rapidly by glucosidases I and II, the post-translational addition of DNJ can cause the accumulation of a mono-glucosylated Tyr(C85S) species capable of binding to lectin chaperones, CNX and CRT (Hebert et al., 1996; Hebert et al., 1995). Indeed, the post-translational presence of DNJ caused the accumulation of monoglucosylated Tyr(C85S), which is monitored by a slight upward mobility shift and an increase in CNX binding (Fig. 3B, compare lanes 9 to 10 and 11 to 12; and data not shown). Furthermore, 62% Tyr(C85S) remained after 3 hours of chase in the presence of DNJ compared to 40% in untreated cells, implicating lectin chaperones in the stabilization of Tyr(C85S).

The effect of the simultaneous inhibition of glucosidases and mannosidases was analyzed. Monoglucosylated Tyr(C85S) was stabilized to 68% in melanocytes incubated with DMJ and post-translational DNJ (Fig. 3B, lanes 11 and 12), supporting the results observed in melanocytes treated with either inhibitor independently. However, only 24% Tyr(C85S) remained in melanocytes treated with DMJ and DNJ prior to translation, a level similar to that observed with DNJ alone (Fig. 3B, lanes 7 and 8).

To examine the fate of tyrosinase further using a more controlled system, TYR(C89R) mRNA was translated in the presence of SP albino melanocytes with or without glucose and mannose analogues, yielding results similar to those seen in intact cells (Fig. 3C, URL). Prevention of mannose trimming by the addition of DMJ stabilized TYR(C89R) from 40% to 68% (Fig. 3C, URL compare lanes 2 and 4). Conversely, translation of TYR(C89R) in the presence of the glucosidase inhibitor or glucosidase and mannosidase inhibitors, led to decreased stability relative to controls (Fig. 3C, URL compare lanes 2, 6, and 8), confirming the role of mannose and glucose trimming in the degradation of Tyr(C85S) observed in intact melanocytes.

Loss of triglucosylated TYR(C89R) is insensitive to protease inhibitors

To examine whether the loss of untrimmed TYR(C89R) remained proteasome-dependent, the protein was translated in the presence of SP albino melanocytes with glucose and mannose analogues and then chased in buffer (Fig. 3C, buffer). A reduction in degradation levels was observed at the end of a 3-hour chase period in untreated cells and in the absence of mannose trimming (Fig. 3C, lanes 2 and 4, compare URL to buffer), supporting a role for mannose trimming in the degradation of TYR(C89R) by the cytosolic proteasome. On the other hand, a decrease in the amount of triglucosylated TYR(C89R) occurred in the absence of cytosol. Untrimmed triglucosylated TYR(C89R) was lost in buffer as in URL, just as untrimmed Tyr(C85S) was degraded in intact melanocytes (compare Fig. 3B, lanes 5 and 6 to Fig. 3C, buffer lanes 5 and

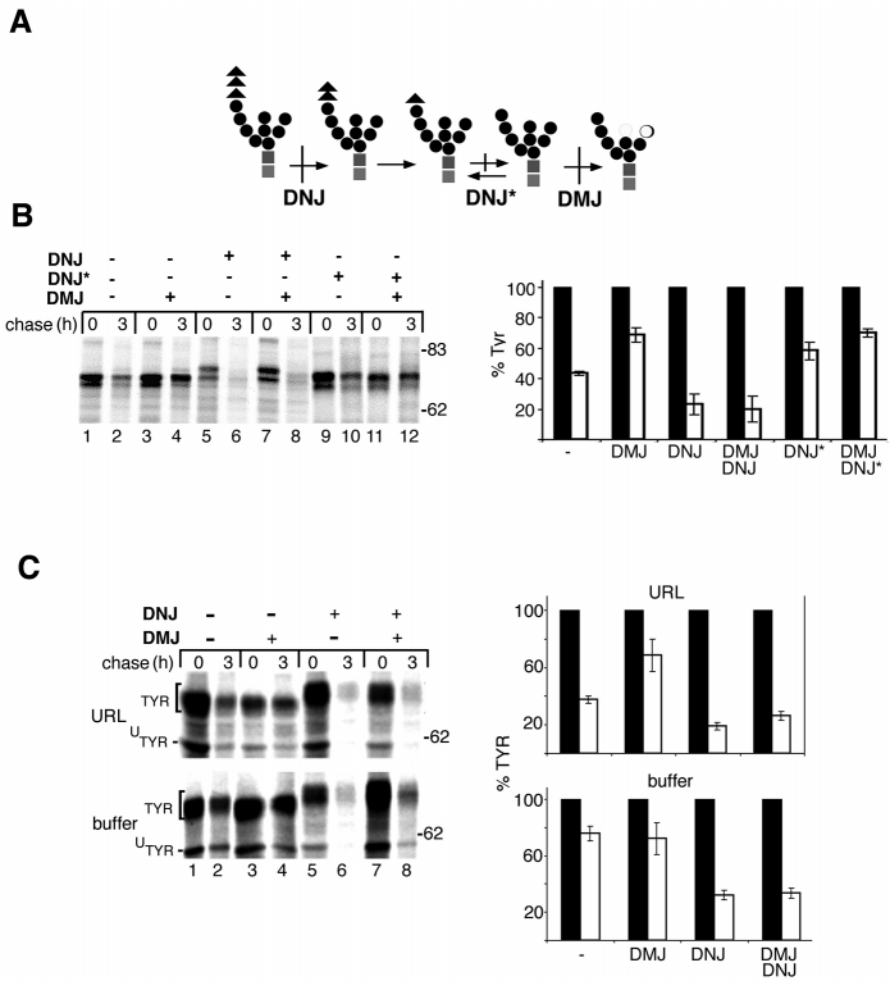

Fig. 3. Mannose and glucose trimming play a role in the degradation of Tyr(C85S). (A) Diagram of the specific effects of the glucose and mannose analogues, n-butyl deoxynanojirimycin (DNJ) and deoxymannojirimycin (DMJ) on the trimming of the $Glc_3Man_9GlcNAc_2$ carbohydrate moiety. Triangles, circles, and squares indicate glucose, mannose, and N-acetyl-glucosamine residues, respectively. (B) Pulse-chase analysis of Tyr(C85S) mouse melanocytes incubated with DNJ as indicated and/or with DMJ throughout starvation, pulse and chase periods, except for samples designated with an asterisk which indicates post-translational addition. 0 and 3 hour time points are indicated in the graph as filled and open bars, respectively. (C) TYR(C89R) mRNA was translated in the presence Tyr(C85S) SP-melanocytes and resuspended in URL or buffer as described previously with or without DMJ and DNJ as indicated.

6). Since proteasomes were not detected after immunoblotting isolated SP-melanocyte membranes with anti-proteasome antibodies (Fig. 2C), our results were not due to contamination by the presence of unwashed cytosolic proteasomes and suggested that a pathway distinct from the proteasome may eliminate triglucosylated tyrosinase.

Targeting to the proteasome or the lysosome are the two main pathways by which proteins are degraded within the cell. To explore how triglucosylated mutant tyrosinase is eliminated, a radioactive pulse-chase analysis was performed in the presence of inhibitors of the proteasome and lysosomal proteases (Fig. 4A). As previously observed in intact melanocytes, the addition of LCT significantly stabilized Tyr(C85S) (Fig. 4A, lanes 13 and 14). In contrast, the concurrent addition of LCT and DNJ led to loss of Tyr(C85S) at the same level as with DNJ alone (Fig. 4A, lanes 3 and 4 versus 9 and 10). Similar results were observed in melanocytes treated with increasing concentrations of LCT or the alternative proteasome inhibitor MG132 (data not shown). A slight decrease in Tyr(C85S) degradation was observed when albino melanocytes were incubated with the lysosomal protease inhibitors, leupeptin and pepstatin (LP), suggesting that the inhibitors were effective and that a small amount of tyrosinase may be degraded by lysosomal proteases (Fig. 4A, lane 6). However, tyrosinase continued to disappear in the presence of LP and DNJ at the same rate as with DNJ alone (Fig. 4A, lanes 7 and 8). Rapid loss was also observed when melanocytes were incubated in LCT, LP, and DNJ (Fig. 4A, lanes 11 and 12). In fact, no significant stabilization of tyrosinase was observed in response to any of the protease inhibitors studied including EDTA, PMSF, and ALLN, which are inhibitors of metallo-, serine, and cysteine proteases, respectively (data not shown).

To investigate whether other untrimmed substrates also disappeared rapidly under the same experimental conditions, we examined the stability of wild-type Tyr in the presence or absence of LCT and DNJ. Degradation of Tyr in the wild-type melanocytes was enhanced by about 30% in the presence of DNJ (Fig. 4B, lanes 1-4). In contrast, the simultaneous inhibition of glucose trimming and the proteasome stabilized wild-type Tyr from approximately 40% to 90% (Fig. 4B, compare lanes 3 and 4 to 7 and 8). Thus, unlike mutant Tyr(C85S), the stability of triglucosylated Tyr in the wild type is sensitive to proteasome inhibitors as viewed by SDS-PAGE.

To confirm the effects of proteasome inhibitors on the loss of triglucosylated TYR(C89R) and to analyze its cytosolic dependence, the human albino TYR(C89R) mutant was incubated in URL or buffer lacking proteasomes in the presence of SP albino melanocytes, DNJ and β-lac (Fig. 4C). The presence of β-lac alone rescued TYR(C89R) as in intact cells, confirming previous observations that trimmed TYR(C89R) degradation occurs largely through the proteasome (Fig. 2B, lanes 7-9 and 4C, URL and buffer, lanes 7-8). However, loss of TYR(C89R) was observed in the presence of DNJ or DNJ/β-lac in both URL and buffer, in agreement with results from intact melanocytes (Fig. 4C, lanes 3-6). Thus, the disappearance of untrimmed mutant TYR(C89R) or Tyr(C85S) does not require the presence of cytosol-containing proteasomes verifying that their disappearance shown in SDS-PAGE analysis is insensitive to protease inhibition.

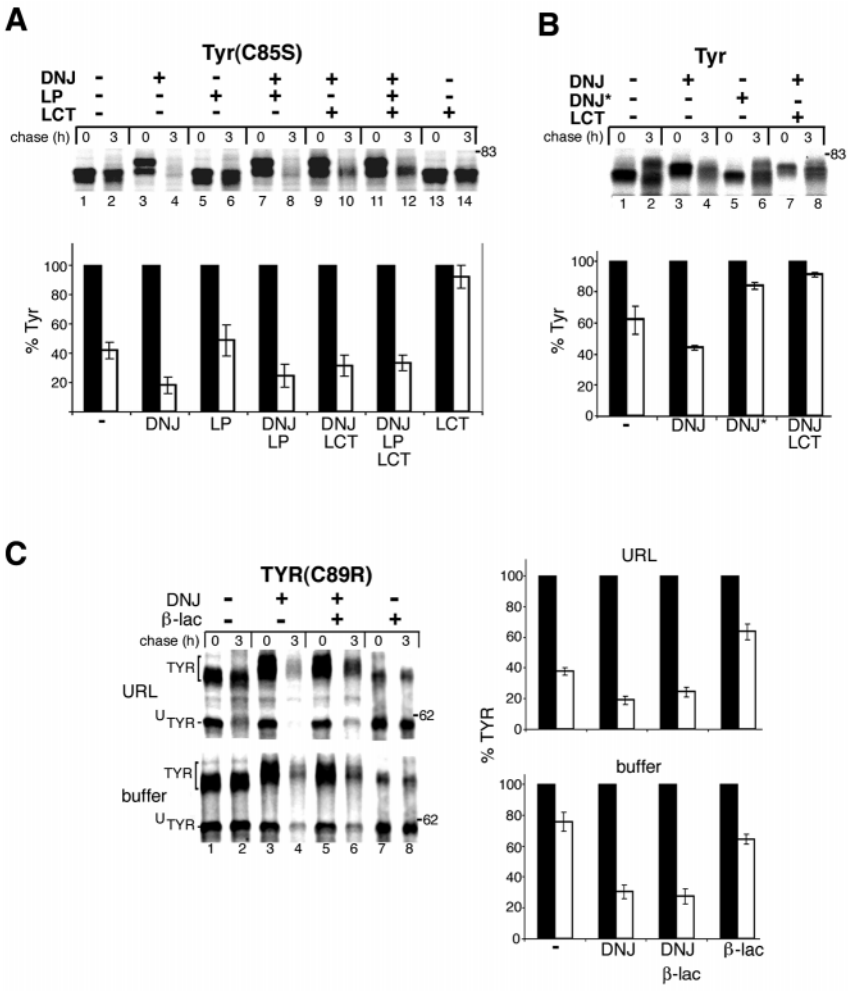

Fig. 4. Loss of triglucosylated Tyr(C85S) is unaffected by known protease inhibitors. (A) Pulse chase analysis of Tyr(C85S) or (B) wild-type Tyr in mouse melanocytes in the presence or absence of the indicated inhibitors throughout the time course, while an asterisk indicates the post-translational addition of the inhibitor. LP indicates leupeptin and pepstatin (10 μg/ml). (C) Human TYR(C89R) was translated in the presence of Tyr(C85S) SP-cells, with or without DNJ and/or β-lac and separated on SDS-PAGE as previously described.

probably representing complexes decorated with multiple chaperones and large Tyr(C85S) aggregates. When DNJ was added, the majority of the protein shifted to the pellet. Moreover, nearly all the triglucosylated Tyr(C85S) appeared on the edge of the stacking gel, suggesting that some of the aggregates could not penetrate the gel. This possibility was also supported by the decrease in tyrosinase detected when melanocytes were incubated with DNJ (Fig. 5, compare Tyr(C85S) in the presence and absence of DNJ). Together, the results indicate that Tyr(C85S) is prone to aggregation, whereas wild-type Tyr is not. The addition of DNJ caused Tyr(C85S), and to some extent Tyr, to aggregate further. Interestingly, tyrosinase migrated as 70-kD and 150-kDa proteins as observed by SDS-PAGE after centrifugation, while the visualized protein probably only represented the smaller aggregates that were discernable by SDS-PAGE. These results indicated that mutant protein underwent aggregation in the absence of CNX and CRT binding.

Untrimmed albino Tyr(C85S) forms ER-localized aggregates, which are degraded by the proteasome

The consistent loss of triglucosylated mouse Tyr(C85S) and human TYR(C89R) in the presence of known protease inhibitors suggests that tyrosinase is degraded by a novel ER-localized proteolytic pathway, or that tyrosinase is present but is not detectable by the methods used. Masking of the required epitope was ruled out because the analysis of tyrosinase stability in SP-melanocytes did not require the use of antibodies. Therefore, we sought to examine the possibility of aggregation as the cause of our inability to detect tyrosinase. To this end, wild-type and albino melanocytes were centrifuged through a 5-25% linear sucrose gradient following incubation in the presence or absence of DNJ. Untreated wild-type Tyr was predominantly in either a ~5S form (monomeric) or in forms ranging from ~6-9S, probably as a result of lectin chaperone binding, as Tyr disappeared from the higher fractions in the presence of DNJ (Fig. 5, Tyr). A small fraction, however, was found in the pellet (P), indicating that some of the wild-type Tyr aggregated in the presence of DNJ.

In contrast, the majority of untreated Tyr(C85S) was observed in forms ranging from ~8 to 10S, in agreement with the prolonged lectin chaperone interactions seen previously (Halaban et al., 2000). In addition, untreated Tyr(C85S) can be detected in forms greater than 10S, as well as in the pellet,

To explore further the extent of mutant triglucosylated tyrosinase aggregation and to determine whether a fraction of the aggregates was too large to penetrate the SDS-polyacrylamide gel, Tyr(C85S) degradation was examined by measuring the production of proteolyzed peptide fragments. This technique, previously used to address the degradation of CFTR, involves monitoring radioactive levels of trichloroacetic acid (TCA) soluble material (Xiong et al., 1999). TCA precipitates intact proteins but not digested products, which are small and remain in the supernatant. Thus, the level of degradation can be determined by measuring the amount of radioactivity present in the supernatant. This methodology retains all the advantages of the SP-melanocyte cell system and permits analysis of protein levels independent of SDS-PAGE or immunoprecipitation.

Human TYR(C89R) was translated in the presence of SP-melanocytes and the indicated inhibitors. After a brief centrifugation to remove untranslocated protein, membranes were resuspended in URL in the presence of an ATP-regenerating system. Membranes were then isolated at successive time points with degradation monitored by both SDS-PAGE and the release of TCA-soluble radioactive material into the supernatant.

Protein levels detected by SDS-PAGE after a 3-hour chase were in agreement with those observed previously (compare

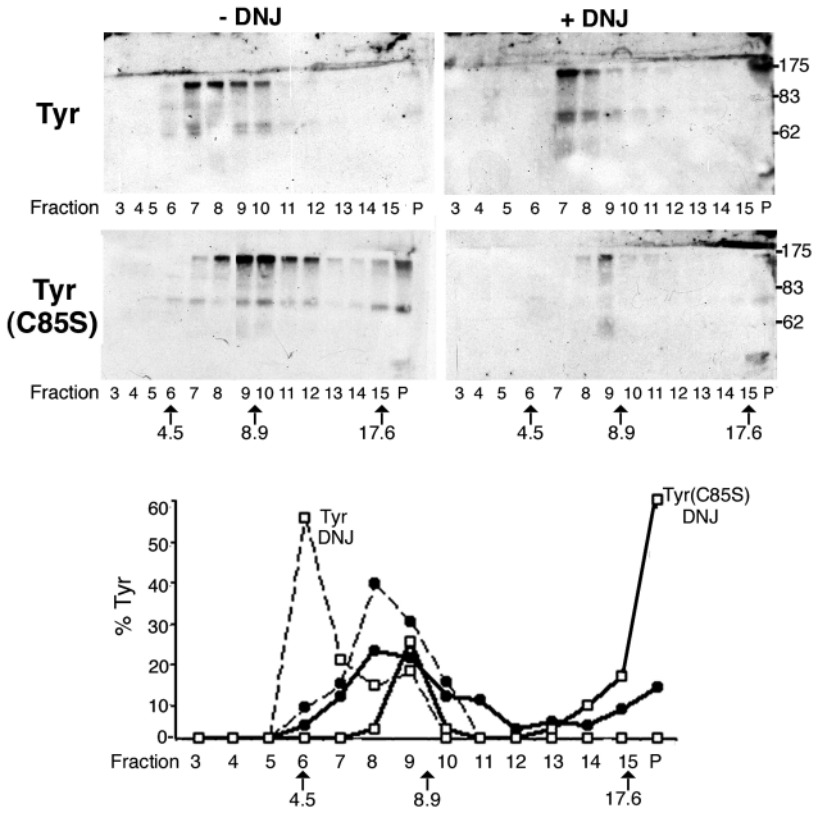

Fig. 5. Albino Tyr(C85S) forms aggregates in the absence of glucose trimming. After a 1-hour incubation in the presence or absence of DNJ, Tyr and Tyr(C85S) melanocytes were lysed in 2% CHAPS, separated on a 5-25% linear sucrose gradient by ultracentrifugation, and subjected to immunoblotting as previously described. The stacking gels were included in the autoradiography.

Fig. 3B, and Fig. 4A with Fig. 6A, SDS-PAGE). After a 5-hour chase, protein levels increased for control, DNJ, and β-lac/DNJ. This repeatable pattern was most striking in samples treated with β-lac/DNJ, where the level of protein increased from 25% to 50% at the end of 3 and 5 hours of chase, respectively. After 8 hours of chase, however, no further increases in protein levels were observed and less than 20% of the initial amount of protein could be detected. In contrast, no increases in protein levels were seen over time and ~50% TYR(C89R) remained in the presence of β-lac. Fluctuations in protein levels suggested the formation of aggregated intermediates, which were eventually, at least in part, disassembled and resolved by SDS-PAGE.

TCA precipitation results showed a different pattern of degradation (Fig. 6A, TCA). First, a steady time-dependent decrease was observed for all samples. Second, protein levels determined by TCA precipitation at 5 and 3 hours were greater than those measured by SDS-PAGE. For example, 70% TYR(C89R) was present after a 3-hour chase in the presence of DNJ (as opposed to 30% determined by SDS-PAGE). Third, TYR(C89R) incubated in the presence of DNJ/β-lac was degraded with kinetics similar to β-lac, not DNJ. Instead, TYR(C89R) incubated with DNJ was degraded at a rate similar to the control. This data implied that TYR(C89R) aggregates eluded detection via SDS-PAGE. However, most of the TYR(C89R) was eventually degraded by the proteasome after 8 hours of chase rather than by an alternative process.

Immunofluorescence microscopy was used to explore protein levels by another method. TYR(C85S) localized to punctate structures within a perinuclear area (Fig. 6B). However, minimal staining was observed when melanocytes were incubated in the presence of DNJ after an 8-hour chase in the presence of cycloheximide. In contrast, the amount of tyrosinase-positive structures as well as the intensity of staining was increased by the addition of the proteasome inhibitor, LCT, in agreement with proteasomal elimination of triglucosylated TYR(C89R).

Untrimmed Tyr(C85S) co-localizes with BiP and PDI

The results of TCA precipitation and SDS-PAGE analysis suggest that aggregates of triglucosylated mutant tyrosinase form before 5 hours of chase but are largely disassembled after 8 hours. To characterize these aggregates and their co-localization with ER-resident proteins, melanocytes were incubated in the presence or absence of DNJ for 5 hours. In melanocytes which contain wild-type Tyr, untreated Tyr appeared as punctate structures located in a perinuclear area that co-localized with the ER marker, BiP, in addition to peripheral areas of the cell body probably corresponding to melanosomes (Halaban et al., 2000). The addition of DNJ had no effect on the morphology of Tyr structures, but did appear to promote the exit of Tyr from the ER as shown by the reduced overlap between Tyr and BiP (Fig. 7A). On the other hand, Tyr(C85S) was concentrated in a perinuclear region, which strongly overlapped with BiP expression, consistent with previous results indicating that it was retained in the ER (Fig. 7B) (Halaban et al., 2000). Conversely, the addition of DNJ to albino melanocytes resulted in the appearance of large clusters, which is likely to correspond to aggregates (Fig. 7B, arrowhead). Such clusters co-localized with BiP and protein disulfide isomerase (PDI), and were not seen in DNJ-treated wild-type Tyr melanocytes. In contrast, the clusters showed limited co-localization with CNX. Elevated concentrations of DNJ increased the number of clusters observed, resulting in some Tyr(C85S) escaping the ER (data not shown).

Aggregation is inhibited under reducing conditions, and enhanced by non-hydrolyzable ATP

To characterize the mechanism of aggregate formation, the stability of human TYR(C89R) was analyzed using the SP-melanocyte system with chases performed in buffer devoid of proteasomes in the presence of DTT, ATP-γ-S or DNJ. The level of aggregation was analyzed by both SDS-PAGE and TCA soluble counts.

After an 8-hour chase, ~40% of TYR(C89R) was aggregated under control conditions (Fig. 8A, lanes 1-4, and Fig. 8B). The amount of aggregation was doubled to approximately 80%

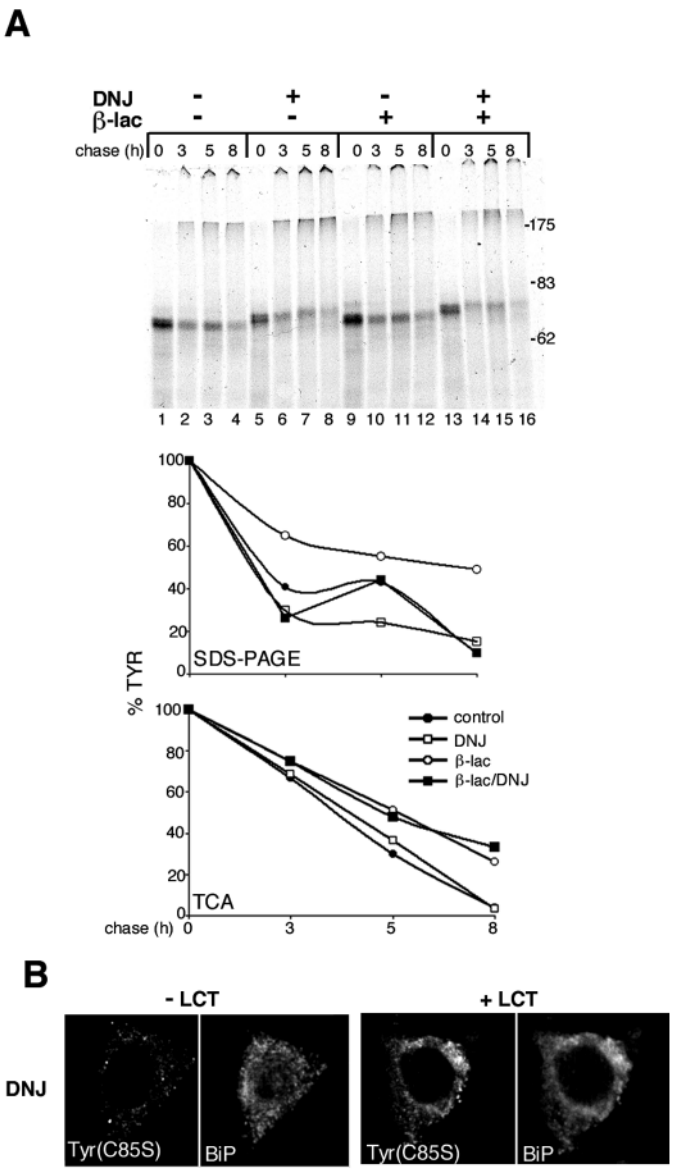

Fig. 6. Triglucosylated TYR(C89R) is degraded by the proteasome at a rate similar to the control. (A) TYR(C89R) was translated in the presence of SP-melanocytes with or without DNJ and/or β-lac for 1 hour. Isolated membranes were resuspended in URL and chased at 37°C. Samples were removed at the indicated chase times, split and either separated on SDS-PAGE as described previously, or precipitated with TCA. The resulting supernatants were quantified using a scintillation counter. (B) Tyr(C85S) melanocytes were incubated with DNJ in the presence or absence of LCT, and chased with the protein synthesis inhibitor cycloheximide for 8 hours. Melanocytes were then fixed, permeabilized, and labeled with anti-tyrosinase or anti-BiP antibodies.

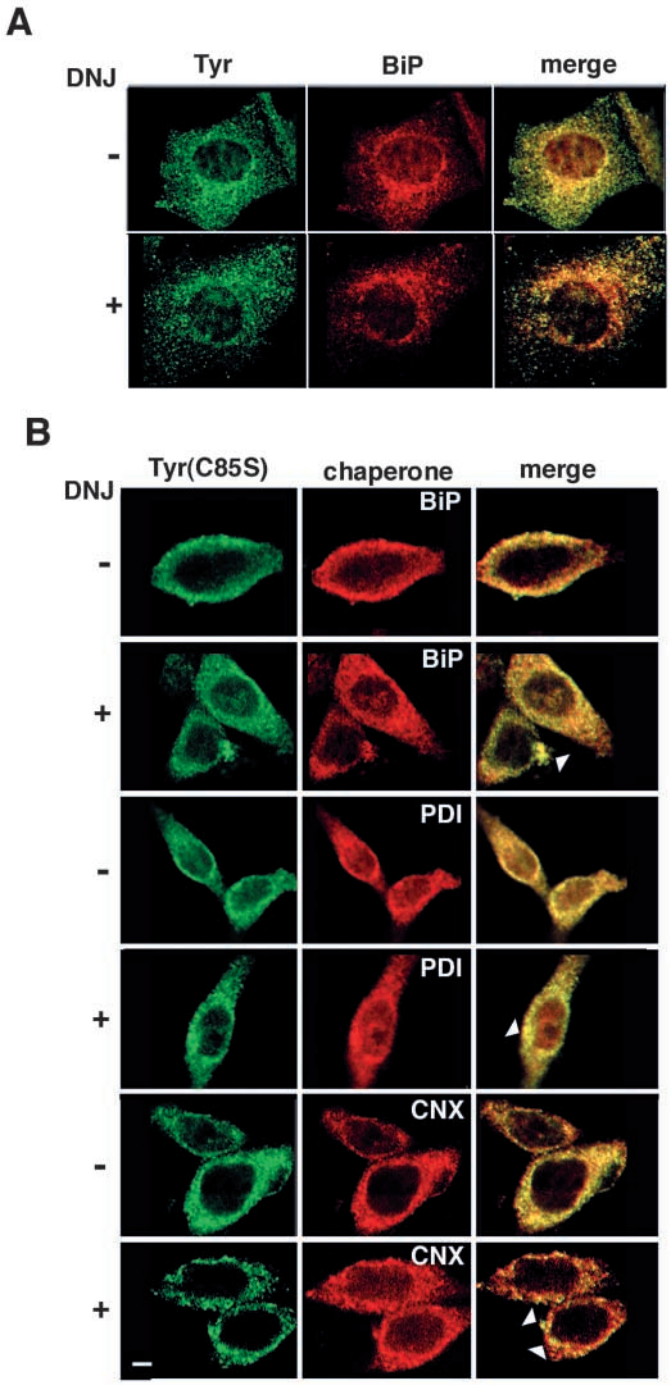

Fig. 7. BiP and PDI, but not calnexin, colocalize with aggregates of triglucosylated Tyr(C85S). (A) After a 4-hour incubation in the presence or absence of 500 μM DNJ, wild-type Tyr and (B) mutant Tyr(C85S) mouse melanocytes were fixed, permeabilized and labeled with anti-tyrosinase (green), anti-BiP, anti-PDI, or anti-calnexin antibodies (red) as indicated. Colocalization is indicated by yellow coloration. Bar, 2 μm.

when DNJ or non-hydrolyzable ATP was present (Fig. 8A, lanes 5-12, and Fig. 8B). This result indicated that lectin chaperones, as well as ATP-dependent chaperones protected tyrosinase from aggregation. Aggregation caused by DNJ could be inhibited by the addition of the reducing agent DTT implicating non-native intermolecular disulfide linkage in the formation of aggregates.

Discussion

A variety of biological systems and methodologies were used to evaluate the role of N-linked glycans in quality control and ERAD allowing for a thorough determination of protein levels.

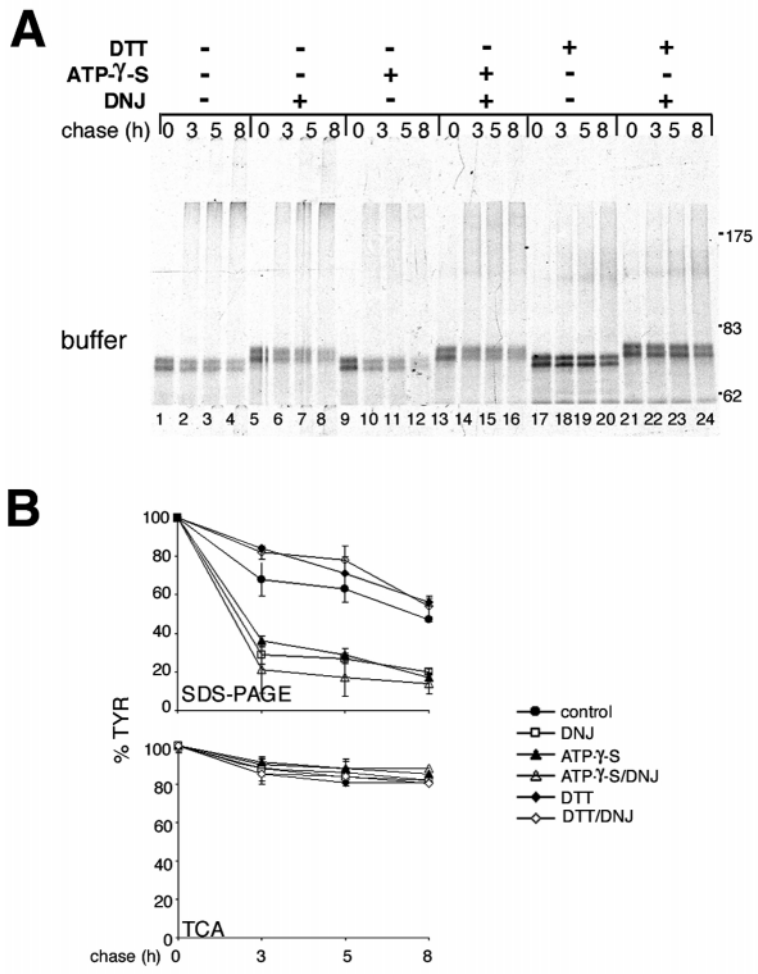

Fig. 8. Aggregation of TYR(C89R) is inhibited by reducing agent and induced by non-hydrolyzable ATP. (A) TYR(C89R) was translated in the presence of SP-Tyr(C85S) melanocytes for 1 hour with or without DNJ or DTT. Membranes were isolated and resuspended in a proteasome-free buffer that contained DNJ, ATP-γ-S or DTT. Samples were isolated at sequential time points and either separated on SDS-PAGE or precipitated with TCA (B).

Glycans were found to act as signals directing the steps in the quality control of tyrosinase. Ablating such signals resulted in the formation of transient aggregates that appeared to contain non-native disulfide bonds, which required ATP hydrolysis for disassembly. These results confirm the significance of N-linked glycans in the maturation and degradation of glycoproteins. The observation that aggregates were eventually eliminated by the proteasome implied that non-carbohydrate specific components of the ER quality-control process could recognize and facilitate the degradation of mutant triglucosylated tyrosinase as if it were a non-glycosylated protein.

We observed that the accumulation of triglucosylated tyrosinase led to enhanced aggregation, suggesting that rapid generation of monoglucosylated glycans was pivotal for the proper maturation of tyrosinase. The monoglucosylated glycans act as a platform for the lectin chaperones CNX and CRT to bind and stabilize the protein (Hammond et al., 1994; Hebert et al., 1995; Ou et al., 1993). This fact is shown here by the stabilization of Tyr in response to glucosidase inhibitors during the post-translational process (Fig. 3B, lanes 9 and 10), as observed also for other proteins (Hebert et al., 1995; Liu et al., 1999; Wang and White, 2000).

Analysis into the mechanism by which mutant TYR(C89R) aggregates implicated erroneous disulfide bond formation. Human tyrosinase has 17 Cys residues with 15 localized to the lumenal mature ectodomain. It is not known how the disulfide bond in tyrosinase forms. The aberrant Cys content of the mutation studied here, found in *Balb c* mice (Tyr(C85S)) and albino humans (TYR(C89R)), may contribute to its susceptibility to aggregation. In this mutant, the absence of the N-terminal Cys residue may disrupt the formation of a disulfide bond, which could be crucial for the stabilization of tyrosinase. Alternatively, the creation of an additional free thiol, the putative partner of Cys85 or 89, may disrupt the concerted folding pathway of tyrosinase by forming non-native disulfide bonds.

Previous studies have established the role of CNX/CRT in promoting correct folding by preventing aggregation and premature degradation of *influenza* hemagglutinin (HA) and MHC Class I heavy chains (Hebert et al., 1996; Vassilakos et al., 1996). CNX has also been found to assist in the proper maturation of tyrosinase (Branza-Nichita et al., 1999; Petrescu et al., 1997; Toyofuku et al., 1999). Here, CNX/CRT did not completely prevent the formation of aggregates, as they occurred with mutant tyrosinase (Fig. 6A, compare SDS-page to TCA). However, binding to lectin chaperones limited the size of the aggregates as they could only be visualized by immunofluorescence microscopy in DNJ-treated Tyr(C85S) melanocytes (Fig. 6B).

Exit of misfolded proteins from the CNX cycle can also be mediated by the activity of ER mannosidase I, which generates the Man8 glycoform (Cabral et al., 2001; Frenkel et al., 2003; Hosokawa et al., 2003; Jakob et al., 1998; Liu et al., 1999). A mannose-trimmed isomer appears to be the substrate for the putative mannose-specific ERAD receptor, EDEM, which targets ERAD substrates for degradation (Hosokawa et al., 2001). Thus, inhibition of mannose trimming should prevent recognition by EDEM and hence, stabilization of the protein. In agreement with this hypothesis, inhibition of mannose trimming through the addition of DMJ enhanced the stability of both Tyr(C85S) and TYR(C89R) (Fig. 3B,C, lane 4) supporting the role of the mannose composition acting as a degradation signal.

In previous studies, the β-site amyloid precursor protein cleaving enzyme, BACE457, disappeared rapidly during simultaneous inhibition of mannose and glucose trimming (Molinari et al., 2003), whereas MHC Class I heavy chains, apolipoprotein A, and cog thyroglobulin were stabilized (Moore and Spiro, 1993; Tokunaga et al., 2000; Wang and White, 2000). Here, concurrent inhibition of both glucose and mannose trimming resulted in a loss of tyrosinase similar to that observed by SDS-PAGE in the absence of glucose trimming alone (Fig. 3B,C, lanes 7 and 8). This was probably due to the lack of sensitivity of SDS-PAGE rather than degradation, as inhibition of glucose trimming enhanced

mutant tyrosinase aggregation. These observations are similar to those reported for BACE457, which formed high-molecular weight complexes prior to degradation (Molinari et al., 2002).

Protein accumulation in the presence of specific protease inhibitors, such as lactacystin, or peptide aldehydes such as MG132 and ALLN, are hallmarks of ERAD substrates (Jensen et al., 1995; Ward et al., 1995). Here, a time-dependent decrease in the level of triglucosylated mutant tyrosinase insensitive to LCT and the depletion of cytosol, was observed by monitoring tyrosinase levels by SDS-PAGE and autoradiography (Figs 3 and 4). These results were not due to incomplete inhibition of the proteasome as the loss also occurred in the presence of SP-cells and buffer devoid of proteasomes (Fig. 2C). One explanation could be the activity of a protease other than the proteasome, such as a lysosomal hydrolase. However, our attempts to identify this protease with inhibitors of known proteases failed (Fig. 4 and data not shown). Alternatively, degradation by a novel proteasome-independent pathway such as reported for α1-antitrypsin variant PiZ (AT PiZ) and lipoprotein lipase (LPL) (Ben-Zeev et al., 2002; Cabral et al., 2000), could have occurred. These previous reports were based on lack of sensitivity to inhibitors of the proteasome and lysosomal hydrolases observed by SDS-PAGE. Interestingly, both of these substrates formed high molecular weight aggregates. In the case of LPL, aggregates did not penetrate the stacker in non-reducing SDS-PAGE (Ben-Zeev et al., 2002). AT PiZ contains a single point mutation that induces the formation of large polymers in hepatocytes (Lomas et al., 1992; Sifers, 1992). On the other hand, under similar conditions, AT PiZ was degraded by the proteasome when expressed in a different cell type (Cabral et al., 2002). However, AT Pi Z did not polymerize in the second cell type as monitored by enhanced secretion. Thus, the possibility of triglucosylated mutant tyrosinase forming insoluble aggregates could explain our observation.

In support of this theory, previous studies show that mutant tyrosinase is misfolded (Francis et al., 2003; Halaban et al., 2000). Results of density gradient centrifugation indicated that control and mutant Tyr(C85S), and to some extent triglucosylated wild-type Tyr aggregated (Fig. 5). Upon closer examination of the aggregates using methods that allowed the monitoring of protein levels independent of SDS-PAGE, we found that aggregates of triglucosylated mutant tyrosinase were transient. The protein levels fluctuated between 3 and 5 hours of chase via SDS-PAGE (Fig. 6A, SDS-PAGE) and it was eventually degraded after 8 hours of chase (Fig. 6A, compare SDS-PAGE and TCA 8 hours). Dissociation of aggregates was sensitive to LCT and its membrane permeable counterpart, β-lac, indicating that they were not diverted to a novel degradative pathway, but rather were degraded by the proteasome.

Although results from sucrose density gradient centrifugation and TCA precipitation indicated that mutant Tyr(C85S) and TYR(C89R), and to some extent wild-type tyrosinase aggregated, such clusters were apparent only by microscopy with mutant tyrosinase when glucose trimming was inhibited. Furthermore, these aggregates were not resistant to degradation. Instead, the data showed that they are dissociated within 8 hours and degraded by ERAD. This result suggested that the large transient aggregates formed as a consequence of masking carbohydrate-specific quality-control signals.

Aggregation appears to represent a cellular strategy for sequestering aberrant proteins until they can be degraded. Cytosolic aggresomes and the ER-localized Russell bodies are relatively long-lived structures degraded by autophagy, a process which involves sequestering defective proteins prior to delivery to the lysosome (Garcia-Mata et al., 2002; Kopito and Sitia, 2000). The aggregates observed here are independent of the cytosol, as they form in SP-melanocytes incubated in buffer (Fig. 3, Fig. 4C and Fig. 8). They are localized to the ER as documented by co-localization with ER-resident proteins, BiP and PDI. Tyr(C85S) did not co-localize with or near the MTOC in the presence or absence of DNJ, suggesting that the tyrosinase-positive structures observed by immunofluorescence were not cytosolic aggresomes (data not shown).

The study of protein misfolding and its consequences has acquired an increase in attention in recent years due to its growing association with a variety of disease states. The use of biological systems that permit thorough monitoring of protein levels can help to provide a clearer picture of the events occurring during protein misfolding, aggregation and degradation. Through the use of semi-permeabilized melanocytes and analysis of protein levels employing methods independent of SDS-PAGE, we have been able to draw a detailed picture of the role of N-linked glycans in the quality control of tyrosinase. Future studies should explore the mechanism whereby mutant tyrosinase aggregates are formed and disassembled. In addition, the reduced cellular systems used in this study will allow further examination and characterization of the quality-control components involved in ER retention and the sorting of defective proteins to the ERAD pathway.

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