Biochemical and Biophysical Research Communications **271,** 22–27 (2000)
doi:10.1006/bbrc.2000.2577, available online at http://www.idealibrary.com on IDEAL®

# Oligosaccharide Trimming Plays a Role in the Endoplasmic Reticulum-Associated Degradation of Tyrosinase

Ye Wang and Matthew J. Androlewicz[1]

*Immunology Program, H. Lee Moffitt Cancer Center and Research Institute, and Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, Florida 33612*

Received March 21, 2000

**The effect of glucosidase and mannosidase inhibitors on the ER-associated degradation of tyrosinase was assessed in transiently transfected COS-7 cells. We found that the glucosidase inhibitors castanospermine and deoxynojirimycin had very little effect on tyrosinase degradation, whereas the mannosidase inhibitors deoxymannojirimycin and kifunensine significantly delayed the rate of tyrosinase degradation as measured by pulse-chase analysis. In addition, we show that tyrosinase degradation is sensitive to the proteasome inhibitor lactacystin and that tyrosinase associates with endogenous calnexin in COS-7 cells. Our data support a model of tyrosinase degradation that involves mannose trimming, calnexin association, and the retrograde transport of tyrosinase from the ER to the cytosol for proteasomal degradation. The pathways of tyrosinase degradation have important ramifications with regard to the exact types of antigenic epitopes that are presented to the immune system.** 

***Key Words:*** **tyrosinase; ER-associated degradation; antigen processing; tumor antigen.**

Quality control mechanisms in the endoplasmic reticulum (ER) play an important part in ensuring that only properly folded and functional proteins are released for further trafficking within the cell. An underappreciated role for ER quality control is to ensure that improperly folded or mutant proteins are efficiently targeted for degradation. This ER-associated degradation (ERAD) was initially presumed to take place within the ER itself, however, more recent evidence suggests that the bulk of ERAD actually occurs in the cytosol and is mediated by the proteasome (for reviews see 1–3). Multiple soluble and membrane-bound glycoproteins have been shown to undergo retrograde transport out of the ER into the cytoplasm where they are degraded. The list includes $\alpha_1$-antitrypsin (4), prepro-$\alpha$ factor (5), carboxypeptidase Y (CPY*) (6), cystic fibrosis transmembrane conductance regulator (CFTR) (7), T-cell receptor subunits $\alpha$ and $\delta$ (8), and MHC class I molecules (9). There is keen interest in elucidating the pathways that govern ERAD. It is currently thought that the translocon serves as the pore through which proteins destined for degradation are transported out of the ER (10). However, very little is known about how proteins are targeted for ERAD. It is known that ER-resident chaperones, such as, calnexin and calreticulin play an intimate role in the proper assembly and folding of newly synthesized proteins (11), and now recent evidence suggests that they may also be involved in targeting the protein for degradation (12). Much of the supporting data has been attained through the use of agents that interfere with carbohydrate trimming, such as, castanospermine and deoxynojirimycin.

The enzyme tyrosinase is one such molecule that undergoes retrograde transport out of the ER for degradation. Tyrosinase is one of the key enzymes involved in melanin biosynthesis, and it is predominantly expressed in melanocytes (13). Under certain conditions, such as in amelanotic melanomas, tyrosinase is rapidly degraded by ERAD (14). It has been shown that this degradation is sensitive to the proteasome inhibitor lactacystin (14, 15). Tyrosinase is also a source of MHC class I-restricted T cell epitopes that play an important role in anti-tumor immunity (16, 17). The retrograde transport of tyrosinase out of the ER plays a critical role in determining precisely what epitopes are generated by the antigen processing machinery in the cytosol. It has therefore become evident that ERAD can play an important role in MHC class I antigen processing and presentation. Other antigens that serve as a source of MHC class I epitopes have

[1] To whom correspondence should be addressed at H. Lee Moffitt Cancer Center and Research Institute, Immunology Program, MRC 4023, 12902 Magnolia Drive, Tampa, FL 33612. Fax: (813) 979-6700. E-mail: androlma@moffitt.usf.edu.

0006-291X/00 $35.00

been reported to undergo a similar pathway of degradation (18, 19).

We set out to further elucidate the mechanisms that control the ERAD of tyrosinase. Previous studies have indicated that oligosaccharide trimming in the ER can have profound effects on protein folding and degradation. For instance, it was shown that glucosidase activity is important for the proper folding and copper loading of tyrosinase (20), and that mannosidase activity is important for the ER-associated degradation of $\alpha_1$-antitrypsin (12). Using the transient expression and degradation of tyrosinase in COS-7 cells as our readout, we found that specific inhibitors of ER mannosidases can substantially delay the degradation of tyrosinase, while inhibitors of ER glucosidases do not. Our results support the hypothesis, as previously reported, that mannose trimming in the ER can have a key regulatory effect on protein degradation (21).

## MATERIALS AND METHODS

*Cell culture.* COS-7 cells (African green monkey kidney cells) were cultured in complete Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY) containing 10% Bovine Calf Serum (HyClone, Logan, UT), 2 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin (Gibco BRL).

*Antibodies.* Rabbit polyclonal antiserum specific for human tyrosinase C-terminal peptide (CEKEDYHSLYQSHL) was generated commercially by Quality Controlled Biochemicals, Inc. (QCB, Hopkinton, MA), and designated 1331. The antibody was affinity-purified using tyrosinase C-terminal peptide coupled to a gel matrix (QCB). The anti-calnexin antibody (Cat. No. SPA-860) was purchased from Stressgen Biotechnologies Corp. (Victoria, BC, Canada).

*Vectors and inhibitors.* The human tyrosinase expression vector pcTYR (22) was a gift from Dr. Ruth Halaban, Yale University (New Haven, CT). The inhibitors, deoxynojirimycin (DNJ), deoxymannojirimycin (DMJ), and castanospermine (CST), were purchased from Sigma (St. Louis, MO). Kifunensine (KIF) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). The proteasome inhibitor lactacystin was purchased from Calbiochem (San Diego, CA).

*Tyrosinase expression and metabolic labeling.* Transient expression of tyrosinase in COS-7 cells was performed following the protocol provided by Qiagen Inc. (Valencia, CA) for use with its transfection reagent Superfect. Briefly, cells were grown to 70–80% confluence in 100-mm dishes, and incubated with a mixture of pcTYR DNA and Superfect (l:5 ratio) for 6 h at 37°C. The dishes were then washed with PBS and incubated in fresh complete medium for 18 h at 37°C. For metabolic labeling experiments, COS-7 cells expressing tyrosinase, were treated with trypsin and harvested. The cells were washed with methionine/cysteine-free medium (RPMI 1640, Gibco BRL), and $2 \times 10^7$ cells resuspended in 500 $\mu$l of labeling media (methionine/cysteine-free medium containing 3% dialyzed fetal bovine serum (dFBS, Gibco BRL). After 1 h preincubation, the cells were labeled with 250 $\mu$Ci/ml [$^{35}$S]methionine/cysteine (Expression Protein Labeling Mix, NEN Dupont, Boston, MA) for 15 min at 37°C. Following the labeling period, the cells were washed with complete medium, resuspended in 1 ml complete medium, and chased for various time points at 37°C. At each time point, the chase medium was removed, and the cells were washed with serum-free medium. The inhibitors were added to the cells during the starving period and were maintained throughout the chase period. The final concentrations of the inhibitors used were: lactacystin (LCT), 50 $\mu$M; deoxynojirimycin (DNJ), 1 mM; deoxymannojirimycin (DMJ), 1 mM; kifunensine (KIF), 100 $\mu$M; castanospermine (CST), 200 $\mu$M.

*Immunoprecipitation.* $^{35}$S-labeled cells were solubilized for 1 h on ice in 500 $\mu$l of TBS (10 mM Tris–HCl and 150 mM NaCl, pH 7.4) containing 1% Triton X-100, 0.5 mM PMSF, and 5 mM iodoacetamide. The lysates were spun at 10,000g for 5 min, and the supernatants were precleared with normal rabbit serum (5 $\mu$l) and protein A-Sepharose (35 $\mu$l) (Sigma) overnight at 4°C. The precleared supernatants were then incubated with tyrosinase antibody 1331 (1:200) or with anti-calnexin antibody (1:200) and protein A-Sepharose for one hour at 4°C. The beads were washed three times with TBS containing 0.5% Triton X-100, and the bound proteins were eluted by boiling in reducing SDS-sample buffer. Immunoprecipitated proteins were then subjected to SDS–PAGE (10%) and phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). Tyrosinase bands were quantified with ImageQuant 5.0 software. Half-life calculations were performed as previously described (23).

## RESULTS

*Site of action of the inhibitors used in this study.* Shown in Fig. 1 is a schematic representation of the N-linked oligosaccharide chain that is added to newly synthesized proteins in the ER, and the sites of action of the inhibitors used in this study. Deoxynojirimycin (DNJ) and castanospermine (CST) are inhibitors of both glucosidases I and II. Deoxymannojirimycin (DMJ) inhibits both ER mannosidases I and II, while kifunensine (KIF) is an inhibitor of ER mannosidase I only. The trimming events depicted are the major steps of carbohydrate processing that occur in the ER, prior to transport of the glycoprotein to the cis Golgi [for review see (24)].

*Glucosidase inhibitors do not affect the degradation of tyrosinase in COS-7 cells.* To assess the role of glucose trimming in the degradation of tyrosinase we performed pulse-chase experiments in transiently-transfected COS-7 cells in the presence and absence of deoxynojirimycin and castanospermine. The cells were preincubated with the inhibitors for 1 h before the initiation of labeling. The results of these experiments are shown in Fig. 2 and Table 1. In untreated cells, tyrosinase degradation occurred with a half-life of ~2 h. In the presence of the two inhibitors, the rate of tyrosinase degradation remained unchanged (Table 1). This can also be seen in both the phosphorimager analysis (Figs. 2A and 2C) and in the quantitative plots of the data (Figs. 2B and 2D).

*Mannosidase inhibitors inhibit the degradation of tyrosinase in COS-7 cells.* In contrast to the glucosidase inhibitors, the specific mannosidase inhibitors deoxymannojirimycin and kifunensine do inhibit the degradation of tyrosinase. The results of these experiments are shown in Fig. 3 and Table 1. The phosphorimager analysis revealed that a greater amount of the tyrosinase was present at the later time points in treated cells (Figs. 3A and 3C), and quantitation of the data clearly shows a delay in the rate of degradation of

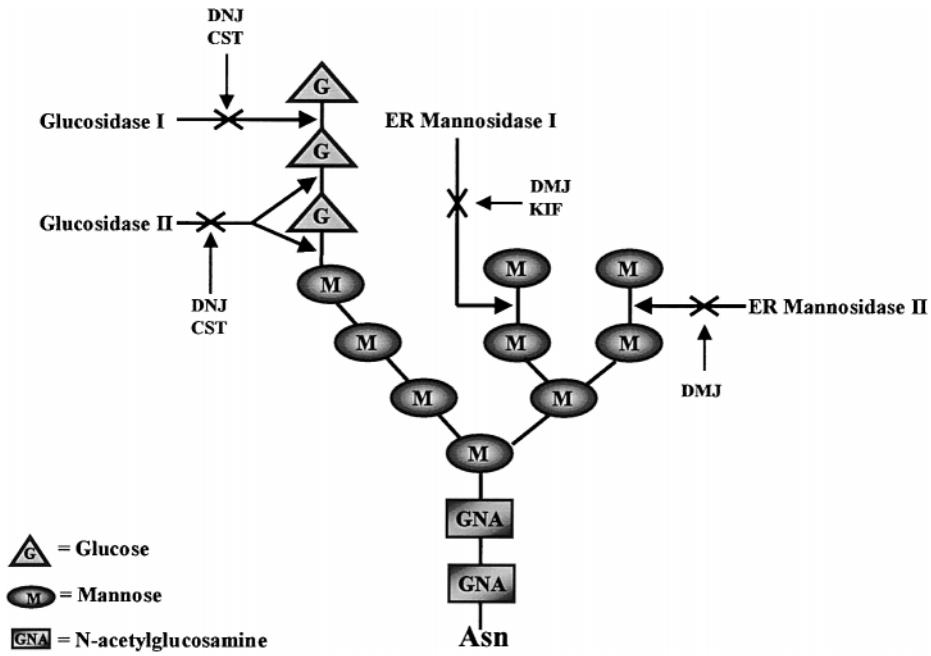


**FIG. 1.** Structure of the N-linked carbohydrate chain and the sites of action of the various inhibitors used. The major oligosaccharide trimming events in the ER and the enzymes involved are shown. DNJ, deoxynojirimycin; CST, castanospermine; DMJ, deoxymannojirimycin; KIF, kifunensine.

tyrosinase in the presence of the inhibitors (Figs. 3B and 3D). The calculated difference in half-life between treated and untreated cells is ~2 h (Table 1). This delay in the rate of degradation is indicative that mannose trimming events are important in controlling tyrosinase degradation.

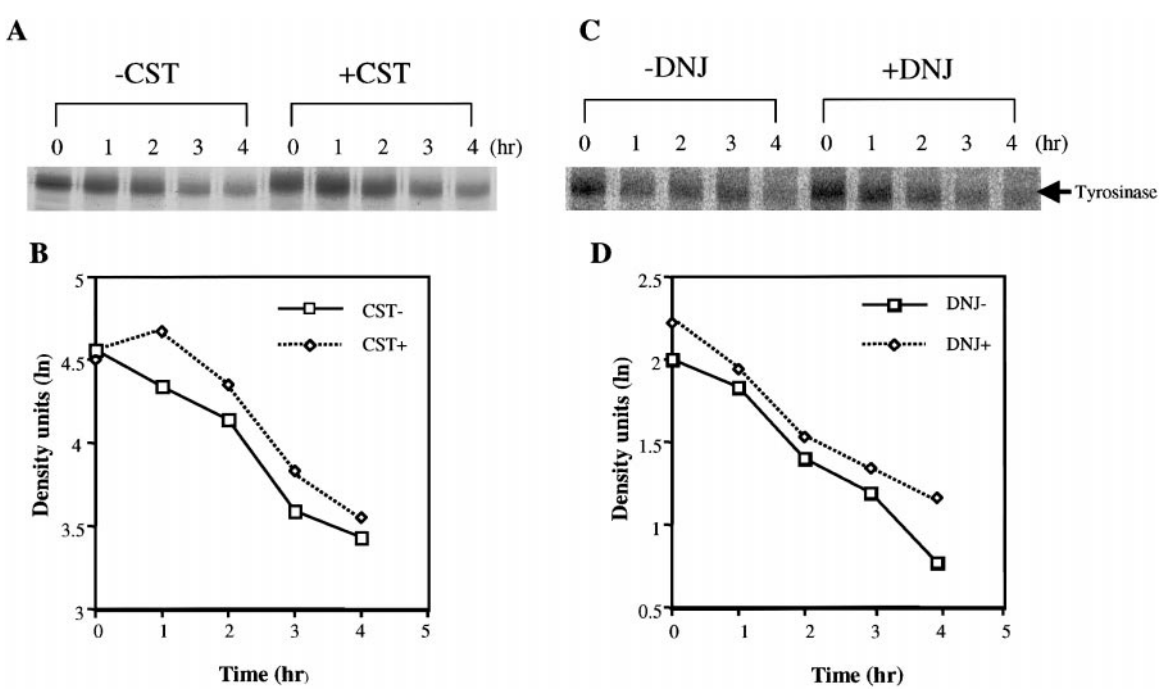


**FIG. 2.** The glucosidase inhibitors castanospermine (CST) and deoxynojirimycin (DNJ) do not affect the rate of tyrosinase degradation. Pulse-chase labeling experiments were performed in transfected COS-7 cells. At the end of each time point the cells were solubilized, and tyrosinase was immunoprecipitated with anti-tyrosinase antibody 1331. Tyrosinase degradation was monitored by phosphorimager analysis of SDS–PAGE gels (A and C), and by plots showing the levels of tyrosinase at each time point in the presence and absence of inhibitors (B and D).

**TABLE 1**

Summary of the Effects of Inhibitors on Tyrosinase Degradation

| | CST | DNJ | DMJ | KIF | LCT |
|---|---|---|---|---|---|
| −Inhibitor | 2.1 | 2.1 | 2.2 | 1.8 | ~2.0 |
| +Inhibitor | 1.8 | 2.1 | 4.3 | 4.4 | 4.7 |

*Note.* The values represent the half-lives of tyrosinase in hours for the experiments shown in this report. Half-life values were determined by the procedure of Chun *et al.* as previously described (23).

*The degradation of tyrosinase is sensitive to the proteasome inhibitor lactacystin.* To confirm that the degradation of tyrosinase in COS-7 cells is mediated through retrograde transport from the ER to the cytosol, we tested whether tyrosinase degradation was sensitive to lactacystin, a specific inhibitor of the proteasome. As shown in Fig. 4, lactacystin had a similar effect as the mannosidase inhibitors. Again, the kinetics were delayed by ~2 h (Table 1). These results confirm that tyrosinase is degraded through a proteasome-sensitive pathway in COS-7 cells, and indicates that tyrosinase is being delivered to the cytosol for degradation. Taken together, the results suggest that mannose trimming in the ER plays a role in delivering tyrosinase to the cytosol.

*Calnexin associates with tyrosinase in COS-7 cells.* To provide support for a role for calnexin in mediating the delivery of secretory proteins to proteasomes as previously suggested by Liu *et al.* (12), we metabolically labeled tyrosinase-expressing COS-7 cells and performed coimmunoprecipitation experiments between calnexin and tyrosinase. The results, shown in Fig. 5, revealed that tyrosinase associated with the endogenous calnexin in COS-7 cells (lane 2). No band corresponding to tyrosinase was seen in association with calnexin in non-transfected COS-7 cells (lane 4). These results indicate that calnexin does associate with tyrosinase in COS-7 cells, as previously reported (25), and supports a role for calnexin in mediating the degradation of tyrosinase.

## DISCUSSION

We have shown that specific inhibitors of ER mannosidases can inhibit the degradation of tyrosinase in COS-7 cells. Our data provide further support for a role for mannose trimming in the regulation of protein degradation in the ER. The current model for the role of mannose residues in ER quality control has been outlined elegantly by Liu *et al.* for the degradation of $\alpha_1$-antitrypsin (21). The basis for this model is the cycle of deglucosylation and reglucosylation by ER glucosidase II and UDP-glucose:glycoprotein glucosyltransferase (UGTR), respectively. Calnexin will bind glycoproteins that contain a single glucose residue, i.e., $Glc_1Man_{7-9}$, but will release the substrate once glucose is cleaved by glucosidase II. While $Glc_1Man_9$ is a good substrate for glucosidase II, $Glc_1Man_7$ and $Glc_1Man_8$

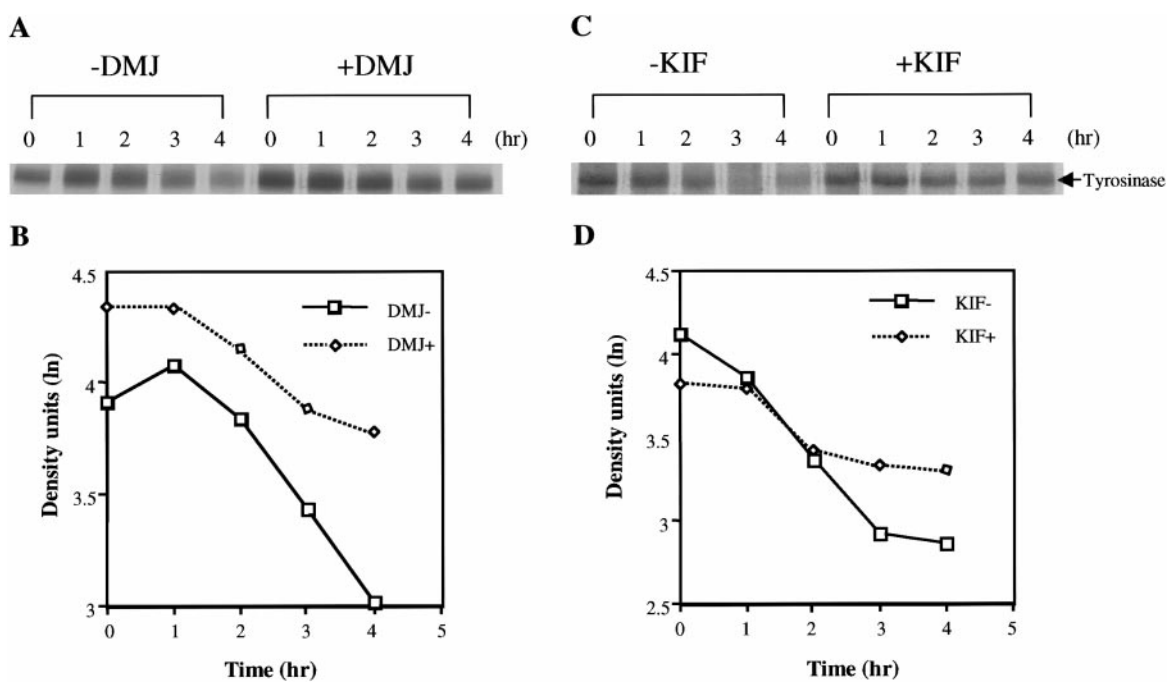


**FIG. 3.** The mannosidase inhibitors deoxymannojirimycin (DMJ) and kifunensine (KIF) delay the rate of tyrosinase degradation. Pulse-chase labeling experiments were performed in transfected COS-7 cells as described in the legend to Fig. 2. The phosphorimager analyses (A and C) and plots (B and D) are shown in the presence and absence of inhibitors.

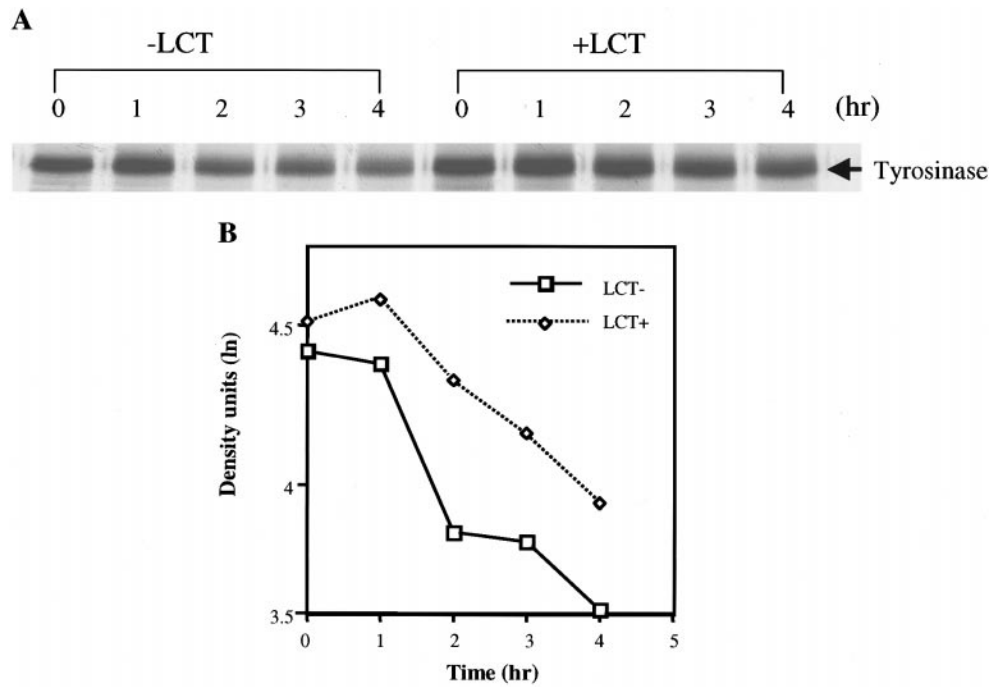


**FIG. 4.** Lactacystin (LCT) inhibits the rate of degradation of tyrosinase. Pulse-chase labeling analysis was carried out in the presence and absence of lactacystin in transfected COS-7 cells. Phosphorimager analysis of the SDS–PAGE gel is shown in A, and the corresponding quantitative plot is shown in B.

are not. Therefore, if glycoproteins are trimmed by mannosidases prior to proper folding then it is likely that they will remain bound to calnexin, ultimately leading to retrograde transport out of the ER and degradation. However, if mannose trimming is inhibited then this leads to increased rounds of deglucosylation/reglucosylation, binding and release by calnexin, and a delay in degradation. The importance of mannose trimming in the degradation of ER glycoproteins has also been shown for the T cell receptor (8). In this study, deoxymannojirimycin was shown to inhibit the ERAD of the T cell receptor δ-chain. It is therefore becoming increasingly clear that the trimming of mannose residues plays an important part in ER quality control.

There is growing evidence of a role for calnexin in the folding of tyrosinase. Two groups have reported an important role for calnexin in promoting the proper folding and copper loading of tyrosinase *in vivo* (25, 20). In these studies, the proper folding of tyrosinase was measured by tyrosinase activity as well as the ability to bind copper. These groups concluded that calnexin was required for the optimal folding of tyrosinase. Our data here suggest that calnexin also plays a role in targeting tyrosinase for degradation if, for some reason, correct folding fails to occur. As a whole, these studies show that calnexin is intimately involved with both tyrosinase folding and degradation. The effect of the mannosidase inhibitors in our system is consistent with a prolonged cycling of tyrosinase between the monoglucosylated and deglucosylated forms, which prevents the targeting of improperly folded tyrosinase for degradation. It is also clear that tyrosinase degradation can occur in a calnexin-independent manner because tyrosinase was still degraded in the presence of glucosidase inhibitors (Fig. 2), which should prevent the association of tyrosinase with calnexin by blocking the trimming of glucose residues down to a single residue. The nature of the calnexin-independent pathway of degradation for tyrosinase remains to be determined. However, a similar pathway has been shown to

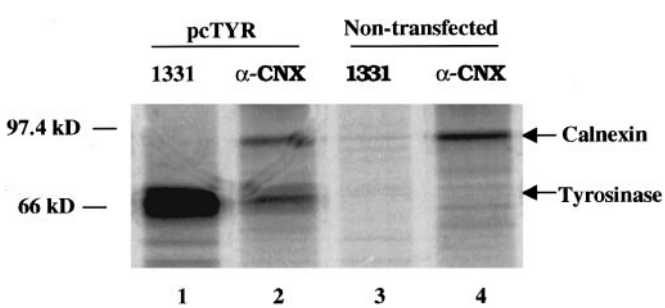


**FIG. 5.** Calnexin associates with tyrosinase in transfected COS-7 cells. Metabolic labeling experiments were performed on tyrosinase-transfected and non-transfected COS-7 cells. After a 15 min labeling period the cells were solubilized and the extracts were immunoprecipitated with either anti-tyrosinase antibody 1331 (lanes 1 and 3) or anti-calnexin (CNX) antibody (lanes 2 and 4). The SDS–PAGE gel was subjected to phosphorimager analysis.

exist for $\alpha_1$-antitrypsin which is non-proteasomal (21). Therefore, based on our results, we would argue that at least two pathways for the ER-associated degradation of tyrosinase exist: one, a cytosolic proteasome-dependent pathway, and two, an ER-lumenal non-proteasomal pathway.

The delineation of tyrosinase degradation pathways has important implications with regard to antigen processing and presentation. The degradation pathways in the cytosol and ER are fundamentally different. While the cytosolic pathway has been well-characterized and is predominantly through the ubiquitin-proteasome pathway, the ER pathway of degradation is not well-defined and is certainly non-proteasomal. It is important to keep in mind that the particular pathway utilized will have a profound effect on the types of epitopes generated for presentation by class I molecules, which, in turn, will affect the overall immune response to tyrosinase. It is therefore essential that the degradation pathways of tyrosinase be further defined. This is especially important with regard to tumor biology and cancer immunotherapy.

## ACKNOWLEDGMENTS

We thank Rick Sifers and Ken Keller for helpful discussions. We acknowledge the use of the Molecular Imaging Core Facility at the H. Lee Moffitt Cancer Center. This work was supported by Grant CA 69115 from the NIH.

## REFERENCES

1. Brodsky, J. L., and McCracken, A. A. (1997) *Trends Cell Biol.* **7,** 151–156.
2. Kopito, R. R. (1997) *Cell* **88**(4), 427–430.
3. Plemper, R. K., and Wolf, D. H. (1999) *Trends Biochem. Sci.* **24,** 266–270.
4. Qu, D., Teckman, J. H., Omura, S., and Perlmutter, D. H. (1996) *J. Biol. Chem.* **271**(37), 22791–22795.
5. Werner, E. D., Brodsky, J. L., and McCracken, A. A. (1996) *Proc. Natl. Acad. Sci. USA* **93**(24), 13797–13801.
6. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) *Science* **273,** 1725–1728.
7. Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L., and Riordan, J. R. (1995) *Cell* **83**(1), 129–135.
8. Yang, M., Omura, S., Bonifacino, J. S., and Weissman, A. M. (1998) *J. Exp. Med.* **187**(6), 835–846.
9. Hughes, E. A., Hammond, C., and Cresswell, P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1896–1901.
10. Wiertz, E. J. H. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996) *Nature* **384,** 432–438.
11. Helenius, A., Trombetta, E. S., Hebert, D. N., and Simons, J. F. (1997) *Trends Cell Biol.* **7,** 193–200.
12. Liu, Y., Choudhury, P., Cabral, C. M., and Sifers, R. N. (1997) *J. Biol. Chem.* **272**(12), 7946–7951.
13. Kwon, B. (1993) *J. Invest. Dermatol.* **100,** 134S.
14. Halaban, R., Cheng, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V., and Hebert, D. N. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6210–6215.
15. Mosse, C. A., Meadows, L., Luckey, C. J., Kittlesen, D. J., Huczko, E. L., Slingluff, C. L., Shabanowitz, J., Hunt, D. F., and Engelhard, V. H. (1998) *J. Exp. Med.* **187**(1), 37–48.
16. Slingluff, C. L., Jr., Hunt, D. F., and Engelhard, V. H. (1994) *Curr. Opin. Immunol.* **6**(5), 733–740.
17. Eynde, B. V. D., and Brichard, V. G. (1995) *Curr. Opin. Immunol.* **7,** 674–681.
18. Selby, M., Erickson, A., Dong, C., Cooper, S., Parham, P., Houghton, M., and Walker, C. M. (1999) *J. Immunol.* **162**(2), 669–676.
19. Ferris, R. L., Hall, C., Sipsas, N. V., Safrit, J. T., Trocha, A., Koup, R. A., Johnson, R. P., and Siliciano, R. F. (1999) *J. Immunol.* **162**(3), 1324–1332.
20. 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.
21. Liu, Y., Choudhury, P., Cabral, C., and Sifers, R. (1999) *J. Biol. Chem.* **274,** 5861–5867
22. Bouchard, B., Fuller, B., Vijayasaradhi, S., and Houghton, A. (1989) *J. Exp. Med.* **169,** 2029–2042.
23. Chun, K., Bar-Nun, S., and Simoni, R. (1990) *J. Biol. Chem.* **265,** 22004–22010.
24. Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54,** 631–664.
25. Toyofuku, K., Wada, I., Hirosaki, K., Park, J. S., Hori, Y., and Jimbow, K. (1999) *J. Biochem. (Tokyo)* **125**(1), 82–89.