Transmembrane domains (TMDs) are known as structural elements required for the insertion into the membrane of integral membrane proteins. We have provided here an example showing that the presence of the TMD is compulsory for the productive folding pathway of a membrane-anchored glycoprotein. Tyrosinase, a type I transmembrane protein whose insertion into the melanosomal membrane initiates melanin synthesis, is misfolded and degraded when expressed as a truncated polypeptide. We used constructs of tyrosinase ectodomain fused with chimeric TMDs or glycosylphosphatidylinositol anchor to gain insights into how the TMD enables the productive folding pathway of the ectodomain. We found that in contrast to the soluble constructs, the membrane-anchored chimeras fold into the native conformation, which allows their endoplasmic reticulum exit. They recruit calnexin to monitor their productive folding pathway characterized by the post-translational formation of buried disulfides. Lacking calnexin assistance, the truncated mutant is arrested in an unstable conformation. We showed that the transmembrane anchor of a protein may crucially, albeit indirectly, control the folding pathway of the ectodomain.

Folding of soluble and membrane-bound glycoproteins of eukaryotic cells begins when the nascent polypeptide chain is translocated into the endoplasmic reticulum (ER) lumen through the translocon pore and is assisted by chaperones. Properly folded proteins achieve an export-competent conformation by folding and refolding in the presence of the chaperone lectins calnexin (CNX) and calreticulin (CRT) and exit the ER (1). Not only the ectodomain of a polypeptide is regulated by the ER quality control but also the folding and assembly of the transmembrane domains (TMDs) in the lipid bilayer (2, 3). However, little is known of the role of the TMD in the folding process of the protein ectodomain. Is the TMD only a structural element required simply to anchor the protein to the membrane or does it also play an active role in the early events during folding within the ER?

To answer this question we took as a model tyrosinase a type I membrane glycoprotein whose maturation in the presence of the ER quality control has been well documented (4–7). Tyrosinase has 533 amino acids, 7 occupied N-glycosylation sites, 17 cysteine residues grouped in 2 cysteine-rich domains, 2 copper binding domains, and 1 C-terminal TMD (8). Tyrosinase folds in the ER and is transported to the trans Golgi network where two copper ions are incorporated (4). From here it continues its journey to the melanosomes where it initiates the melanin synthesis. This protein is a melanoma antigen and an important target for anti-melanoma vaccine therapies. Hence, understanding the role of the molecular determinants of tyrosinase for its processing is important for the design of therapeutic approaches based on tyrosinase mutants that are good candidates for the efficient production of antigenic peptides. It has been reported that a truncated tyrosinase mutant, lacking the TMD and cytoplasmic tail, is retained in the ER by calreticulin and BiP and degraded by the ER-associated degradation (ERAD) pathway (9, 10).

To understand how the TMD of tyrosinase can determine the productive folding and maturation of tyrosinase, we have analyzed chimerical proteins in which the TM domain of tyrosinase has been replaced by a variety of TMDs and a glycosylphosphatidylinositol (GPI) anchor. We found that unlike soluble tyrosinase, tyrosinase chimeras tethered to the membrane either by a GPI anchor or by a variety of other TMDs acquire a transport-competent state and exit the ER. Productive folding is associated with calnexin binding, which is restored for all membrane-anchored tyrosinases. The role of the TMD is to enable a proper calnexin interaction that determines a productive folding pathway of tyrosinase.

**MATERIALS AND METHODS**

**Reagents, Cell Lines, and Antibodies**—Human embryonic kidney (HEK) 293 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Cells were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Sigma), 50 units/ml of penicillin, and 50 mg/ml of streptomycin (Invitrogen) and maintained at 37 °C with 5% CO₂. Rabbit anti-calnexin antiserum was a kind gift from Dr. J. J. Bergeron (McGill University, Canada). T311 (NeoMarkers, Fremont, CA) is a monoclonal antibody IgG2a recognizing human tyrosinase.
TABLE 1
List of primers used in this report
F-forward, R-reverse.

| Name           | Sequence 5′-3′                                                                 |
|----------------|-------------------------------------------------------------------------------|
| P1. ST_TM stop(bold) mutagenic primer | pGCTGCTCTTGAAGCTCACAAAGG |
| P2. SwaI/SfuI (bold) selection primer | pGCTGCTCTTATTACTGCGAATTTCAGAATTAAAA|CTTC |
| P3. ST_GPI F   | CTCCAGGACGCAGCCACACCGGGGCCGCG |
| P4. ST_GPI R   | CTCCAGGAGATCGTGAGCTGACGTCTTC |
| P5. ST_MU F    | CTCCAGGATTTATTCTGCAATGCTAC |
| P6. ST_MU R    | CTCCAGAATCCCTCCTTTCTCTTCAACAC |
| P7. ST_E2 F    | CTCCAGGCGCTCTGCGCATATTAGTGA |
| P8. ST_E2 R    | CGGAGATCCAGATATACCAGG |
| P9. ST_405 F   | CTCCAGATGCTGGTCTTTGGAAGCC |
| P10. ST_405 R  |                                                                                     |

Human Tyrosinase Chimeras—The RPTPaseMu and miniPLAP templates were kind gifts from Dr. Stefan Szedlacek (Institute of Biochemistry, Bucharest, Romania) and Dr. Neil Bulleid (University of Manchester, Manchester, UK) respectively. The framework for all tyrosinase chimeras was the pTriEx-1.1-ST construct obtained as previously described (10) by cloning soluble tyrosinase in-frame with the 8 histidines of pTriEx-1.1(Novagen EMD Biosciences). The different transmembrane domains were amplified by PCR (High Expand Polymerase kit; Roche Applied Science) using the primers and templates listed in Table 1. A Xhol restriction site was introduced at the 5′-end and BglII and Xhol restriction sites at the 3′-end (New England Biolabs). The PCR products were cloned in pGem-T-Easy vector (Promega), digested with Xhol, and cloned in the Xhol site of pTriEx-1.1_ST. BglII digestion was used to determine the orientation of the cloned fragment. This resulted in the creation of three constructs, pTriEx-1.1-ST_Mu (Table 1, P5, P6), pTriEx-1.1-ST_E2 (Table 1, P7, P8) and pTriEx-1.1-ST_GPI (Table 1, P3, P4).

The deletion of the tyrosinase cytosolic tail (the pTriEx-1.1-ST_TM construct) was accomplished using a site-directed mutagenesis kit (BD Biosciences, Clontech) following the manufacturer’s instructions. The mutagenic primer (Table 1, P1) mutates Cys-500 to a STOP codon. The selection primer mutates the unique restriction site SfuI (ATTTAAATCCTC) to SwaI (TTCGAA) restriction site. ST_405 was cloned in pcDNA3.1/V5-His A (Invitrogen) in BamHI/Xhol restriction sites using the primers P9 and P10. All primers are listed in Table 1.

Transfection of Cells and Metabolic Labeling—Constructs were transiently transfected in HEK293 cells. Semiconfluent cells (50–60% confluency) 24 h post-seeding in 6-well dishes were used to transiently express tyrosinase cDNAs (3 mg of DNA/well) using polyethylenimine (PEI) solution (1 mg/ml, pH 8) (6 μl of PEI/well). Cells were analyzed 24 h after transfection. For metabolic labeling, transfected cells (~3 × 10⁶ cells) were starved in the cysteine-/methionine-free medium for 1 h, pulse labeled with 100–150 μCi of [³⁵S]methionine/cysteine for 5 or 20 min, and chased for the times specified. Immediately after the chase, cells were harvested in cold PBS. Cells were then lysed with CHAPS lysis buffer (50 mM HEPES buffer, pH 7.5, containing 2% CHAPS, 200 mM NaCl, and 0.5% protease inhibitor mixture containing leupeptin, aprotinin, sodium EDTA, bestatin, 4-(2-aminoethyl)benzenesulfonylfluoride, and E-64) for 1 h on ice. In some experiments, 5 mM DTT was added to the cell culture medium at the end of the chase period. After 5 min cells were harvested in PBS containing 20 mM N-ethylmaleimide to alkylate the free SH groups and then lysed as described above.

Immunoprecipitation and SDS-PAGE—³⁵S-labeled cell lysates were centrifuged, and supernatants were incubated with T311 antibodies (1:50) overnight at 4 °C, followed by the addition of 20 μl of protein A-Sepharose and further incubation for 1 h at 4 °C. The slurry was washed three times with 0.5% CHAPS in HEPES buffer. Tyrosinase was eluted by boiling the slurry for 5 min in SDS sample buffer with 5% 2-mercaptoethanol. In some experiments, tyrosinase was eluted from protein A with 1% SDS and the protein was resolved by SDS-PAGE under non-reducing conditions, i.e. in the absence of 2-mercaptoethanol. Chaperone co-immunoprecipitations were performed as described previously (10). Briefly, lysates were immunoprecipitated with anti-calnexin, and the washed slurry was eluted with 1% SDS, diluted ten times with lysis buffer, and reprecipitated with T311 antibodies. The bound proteins were eluted in reducing conditions and resolved by SDS-PAGE. The gels were visualized by autoradiography. Relevant bands were quantified by scanning densitometry.

Immunofluorescence—HEK293 cells were plated on coverslips and transfected with different constructs using the methods described above. After 24 h the cells were rinsed with PBS and either fixed with paraformaldehyde (PFA) for 20 min at room temperature or fixed and permeabilized with methanol at −20 °C for 5 min. After washing three times in PBS, cells were incubated with the primary antibody, T311 (1:250), diluted in PBS for 30 min at room temperature. Following three washes with PBS they were further incubated with the appropriate Alexa 594-conjugated secondary antibodies (1:400) in PBS for 30 min at room temperature. Finally, cells washed three times with PBS were mounted on coverslips in Vectashield mounting medium (Vector Laboratories) and viewed with a Nikon Eclipse E 600 fluorescent microscope. Images were processed using Adobe Photoshop 5.0 software.

RESULTS

Constructs—To address the requirements for membrane tethering in the folding and maturation of soluble tyrosinase, tyrosinase chimeras in which the ectodomain is attached to the membrane were designed. A number of hydrophobic membrane insertion sequences and a GPI anchor were used (Fig. 1). The first construct, pTriEx-1.1-ST_TM, was a tyrosinase mutant including the ectodomain and the tyrosinase TMD but lacking the cytoplasmic tail. Two soluble tyrosinases were
Transmembrane Domain Role in Ectodomain Folding

![Schematic representations of the constructs. A, linear structure of wild-type tyrosinase. The positions of the TM domain (475–499), of the N-glycans, and of the cytoplasmic tail (C) are shown. B, ST_TM protein. This protein differs from WT in that the C domain is absent. C, structure of ST protein, which has the ectodomain of tyrosinase but lacks the TMD and C domain. D, ST_405 protein. A shorter variant of ST truncated after the amino acid 405. E and F, ST_Mu and ST_E2 fusion proteins. Chimeras were constructed in which tyrosinase ectodomain was fused with the TMD of RPTPaseMu (E) and HCV E2 envelope protein (F), respectively. G, ST_GPI fusion protein. The TMD of tyrosinase was replaced with a GPI anchor. The sequences of the wild-type TM, RPTPaseMu TM, and HCV TM are shown.](image)

FIGURE 1. Schematic representations of the constructs. A, linear structure of wild-type tyrosinase. The positions of the TM domain (475–499), of the N-glycans, and of the cytoplasmic tail (C) are shown. B, ST_TM protein. This protein differs from WT in that the C domain is absent. C, structure of ST protein, which has the ectodomain of tyrosinase but lacks the TMD and C domain. D, ST_405 protein. A shorter variant of ST truncated after the amino acid 405. E and F, ST_Mu and ST_E2 fusion proteins. Chimeras were constructed in which tyrosinase ectodomain was fused with the TMD of RPTPaseMu (E) and HCV E2 envelope protein (F), respectively. G, ST_GPI fusion protein. The TMD of tyrosinase was replaced with a GPI anchor. The sequences of the wild-type TM, RPTPaseMu TM, and HCV TM are shown.

Designed, pTriEx-1.1-ST, truncated at the 474 codon (10), and pcDNA3.1-ST_405, truncated upstream at codon 405. In the next construct, pTriEx-1.1-ST_Mu, the tyrosinase TMD was replaced with the TMD of receptor-like protein tyrosine phosphatase Mu (RPTPaseMu), which is known to localize to the plasma membrane (11). Another construct (pTriEx-1.1-ST_E2) was designed in which the ectodomain was attached to the TMD of the HCV E2 protein, which was reported to contain ER retention signals (12). Finally, a GPI-anchored construct (pTriEx1.1-ST_GPI) was designed in which the ectodomain was fused with a GPI anchor.

The TMD Is Sufficient to Restore Calnexin Interaction and ER Export of Truncated Tyrosinase—To determine whether the TMD alone is sufficient to maintain tyrosinase in a transport-competent state, we constructed a tyrosinase truncation mutant, ST_TM, consisting of the ectodomain and the TMD and lacking the cytoplasmic domain. HEK293 cells were transfected with the pTriEx1.1-ST_TM construct and analyzed by Western blot (Fig. 2A). Wild-type tyrosinase and soluble tyrosinase transfected in the same cell line were used as controls. As seen in Fig. 2A, complex or hybrid N-glycans were identified in the Endo H digests, indicating that the ST_TM tyrosinase mutant exits the ER and travels through the Golgi. Complex N-glycans were found in the control wild-type tyrosinase and were absent in soluble tyrosinase (Fig. 2A).

To confirm their passage through the secretory pathway, the localization of the mutant and wild-type tyrosinasises was compared in an immunofluorescence experiment. Transfected HEK293 cells were either fixed with PFA or permeabilized with methanol and stained with anti-tyrosinase antibodies. As seen in Fig. 2B, ST_TM localized to the plasma membrane in non-permeabilized cells. This is a consequence of the signal deletion in the cytoplasmic tail that normally targets WT tyrosinase to late endosomal compartments (13). In contrast, WT staining in PFA-fixed cells was less pronounced, in agreement with its predominant intracellular localization (Fig. 2B). ST was considered a negative control for plasma membrane localization due to its predominant ER localization.

To analyze whether the absence of the cytoplasmic tail would have an adverse effect on the interaction of ST_TM with calnexin, the association of ST_TM, WT, and ST with the ER chaperone calnexin in transfected cells was investigated by pulse-chase and co-immunoprecipitation with anti-calnexin and anti-tyrosinase antibodies (Fig. 3A). Although soluble tyrosinase interacts very poorly with calnexin, both wild-type and ST_TM tyrosinase associated with the lectin chaperone (Fig. 3A). The interactions had similar kinetics with a half-life of 30 min and a gradual decrease during the chase (Fig. 3B). Further, we checked whether the association with calnexin was due to the specific binding to the monogalactosylated N-glycans by performing a co-immunoprecipitation experiment in the presence of N-butyl-deoxynojirimycin, which abolishes the interaction of CNX with the N-glycans. As seen in Fig. 3C, a 95% decrease in the association of calnexin with WT and ST_TM was noticed under these conditions. As expected, ST did not bind calnexin in the presence of NB-DNJ either.

To determine whether the location of the truncation site could affect the properties of the tyrosinase ectodomain, another soluble mutant, ST_405, truncated at the codon 405, situated downstream the second copper domain, was analyzed. We found that similar to the ST that was truncated in the vicinity of the TMD, ST_405 was expressed as a high mannose glycosylated form. To determine whether the addition of the cytoplasmic tail would have an adverse effect on the interaction of ST_TM with calnexin, the association of ST_TM, WT, and ST with the ER chaperone calnexin in transfected cells was investigated by pulse-chase and co-immunoprecipitation with anti-calnexin and anti-tyrosinase antibodies (Fig. 3A). Although soluble tyrosinase interacts very poorly with calnexin, both wild-type and ST_TM tyrosinase associated with the lectin chaperone (Fig. 3A). The interactions had similar kinetics with a half-life of 30 min and a gradual decrease during the chase (Fig. 3B). Further, we checked whether the association with calnexin was due to the specific binding to the monogalactosylated N-glycans by performing a co-immunoprecipitation experiment in the presence of N-butyl-deoxynojirimycin, which abolishes the interaction of CNX with the N-glycans. As seen in Fig. 3C, a 95% decrease in the association of calnexin with WT and ST_TM was noticed under these conditions. As expected, ST did not bind calnexin in the presence of NB-DNJ either.

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Interestingly, in non-reducing conditions we could identify indicating that folding occurs in the first 8 min of pulse.

There is a gradual increase in the appearance of the DTT-resistant intermediate in the first 20 min of synthesis, which indicates the formation of inaccessible, buried disulfide bridges. The conformation of the DTT-resistant tyrosinase is more compact than the one of the DTT-sensitive form, hence it migrates faster in non-reducing gels. In contrast to WT migrating with a mobility that increases during the chase period, the same migration pattern is maintained throughout the chase period of the ST (Fig. 4B).

To determine whether these ST intermediates are sensitive to DTT, we have run the treated samples in reducing and non-reducing conditions on the same gel (Fig. 4C). Because at 0 min chase point the DTT-treated sample migrates with a similar velocity in non-reducing and in reducing conditions, ST intermediates appear to be DTT sensitive. WT tyrosinase treated with DTT migrates with the same velocity as the reduced tyrosinase at 0 chase time and with faster mobility at 12 min chase (Fig. 4C), confirming the data from Fig. 4B. Unlike WT tyrosinase, the soluble form follows a different folding pathway characterized by the formation of intermediates in which the S–S bonds are always solvent accessible. In the absence of calnexin, the protein is driven into an unproductive folding pathway characterized by disturbed kinetics of S–S bond formation.

Based on the assumption that folded proteins are stabilized by disulfide bridges that are buried once the protein folds into the native structure, soluble tyrosinase is a protein with a non-native conformation characterized by disulfide bridges susceptible to low concentrations of reducing agents. Hence, the experiments characterizing the in vivo DTT sensitivity of WT and ST have shown that in the absence of the TM domain, the ectodomain of tyrosinase is unable to acquire a stable conformation.

**Transmembrane Domain Role in Ectodomain Folding**

**Kinetics of Disulfide Bond Formation Differ between Wild-type and Soluble Tyrosinase**—Next, we studied the effects of preventing the interaction with calnexin on the conformation of the ectodomain. The differences between the folding pathways of membrane-bound and soluble tyrosinase were examined by assessing the accessibility of disulfide bonds to DTT. HEK cells were transfected with either pTriEx-1.1-WT or pTriEx-1.1-ST. The transfected cells were pulsed for 8 min, chased for 1 and 12 min, and incubated or not with 5 mM DTT before harvesting (Fig. 4). This DTT concentration has been shown before to cleave only exposed disulfide bridges found on incompletely folded chains, whereas folded polypeptides were unaffected (26). Pelleted cells were alkylated with 20 mM N-ethylmaleimide to prevent further oxidation and folding, lysed, and immunoprecipitated with the T311 antibody. As seen in Fig. 4A, at 0 min chase, both WT and ST run as a main band (WT-ox and ST-ox) with a slightly faster migration in non-reducing than in reducing conditions (WT-red and ST-red). This migration velocity does not change for either of the two tyrosinases during the chase, indicating that folding occurs in the first 8 min of pulse. Interestingly, in non-reducing conditions we could identify aggregates and possibly oligomers of WT and ST that are linked by interchain disulfide bridges, because they disappear in reducing conditions.

As seen in Fig. 4B, a different pattern is observed when the WT samples were treated with DTT. Whereas a slower migrating band (DTT-sensitive) predominates at 0 and 1 min chase, a faster migrating band (DTT-resistant) prevails at 12 min chase.

**The Productive Maturation of Tyrosinase Is Independent of the TMD Amino Acid Sequence**—The results obtained so far established that in the absence of the TMD the ectodomain is unable to interact with calnexin, leading to unproductive folding that results in an incompletely folded protein. Next, the potential impact of the composition and length of the transmembrane anchor on the maturation of the tyrosinase ectodomain was investigated. As described above, tyrosinase chimeras with the luminal domain of tyrosinase and the TM domain of RPTPaseMu (ST_Mu) or of the HCV E2 envelope glycoprotein (ST_E2) were constructed (Fig. 1). Because the cytoplasmic tail is not involved in the ER maturation process, the constructs are devoided of this domain. Endo H digestion of transfected cells showed that ST_Mu acquired Endo H-resistant glycans consistent with the exit of ST_Mu from the ER (Fig. 5A). Because the TMD of the E2 protein contains ER retention signals, the
ST_E2 chimera was kept within the ER, as shown by its Endo H pattern. The subcellular localization investigated by immunofluorescence experiments confirmed that ST_Mu reached the plasma membrane whereas ST_E2 was retained in intracellular membranes (Fig. 5B).

The calnexin co-immunoprecipitation of the two mutants in pulse-chased cells revealed patterns of association similar to those of WT and ST_TM (Fig. 5C). The half-life of the calnexin interaction with ST_Mu was slightly shorter than for the other mutants (20 min compared with 30 min; Fig. 5D). However, the association with CNX exhibited the same kinetics.

These data suggest that the interaction of calnexin with tyrosinase is independent of the TMD composition. Together with the results shown in Fig. 3C showing that the interaction is almost completely inhibited in the absence of specific N-glycans, these results strengthen the idea that the composition of the TMD does not directly influence the ER quality control of the tyrosinase ectodomain.

The GPI Anchor Restores the Interaction of Soluble Tyrosinase with Calnexin—Because the TM anchor sequence is not a prerequisite for the calnexin interaction, it was hypothesized that preserving the WT topological space would enable the productive maturation of the tyrosinase ectodomain. A chimera was constructed by attaching the GPI anchor signal from human placental alkaline phosphatase (PLAP) to the tyrosinase ectodomain (ST_GPI) (Fig. 1).

Endo H digestion of the lysates of ST_GPI-transfected cells revealed the processing of the GPI construct glycans to Golgi-processed oligosaccharides (Fig. 6A). The expected ST_GPI localization at the plasma membrane was observed by immunofluorescence staining in a similar experimental setup as described in the previous sections (Fig. 6B).

The pull down of ST_GPI associated with calnexin in metabolically labeled pulse-chased cells showed that the presence of a GPI anchor was sufficient to restore the interaction of the ectodomain with calnexin with a half-life of 20 min (Fig. 6, C and D). These data show that a GPI anchor can substitute for the TMD and is capable of restoring the interaction of the tyrosinase ectodomain with calnexin with similar kinetics and half-life as WT tyrosinase (Fig. 3, A and B).

**DISCUSSION**

We have used wild-type, truncated, and chimeric tyrosinase molecules to characterize the importance of the TM region for the folding pathway of the tyrosinase ectodomain. We showed that the tyrosinase folding pathway includes at least two intermediates and that the formation of the export-competent, DTT-resistant conformation is highly regulated by the TMD. Indeed, in the absence of the TMD, the ectodomain is arrested in a DTT-sensitive conformation that determines its ER retention and targeting to degradation.

The DTT sensitivity experiments show that there is a direct correlation between the calnexin interaction and the formation of critical disulfide bonds in tyrosinase that engage the polypep-
tide chain in a productive folding pathway. Wild-type tyrosinase folds co-translationally into a non-native DTT-sensitive conformation. Only post-translationally the polypeptide chain reaches the native conformation, characterized by the presence of DTT-resistant disulfides. It has been documented that in the absence of CNX or specific N-glycans tyrosinase displays a non-productive folding pathway (7, 14). Similarly, the truncated chain is arrested in the co-translationally acquired non-native conformation. Although the formation of S–S bonds starts co-translationally (15), the generation of native disulfides requires a post-translational step (this report). This step occurs after the polypeptide release from the translocon channel at the ER membrane in the case of WT, or in the ER lumen for soluble tyrosinase. Hence, the efficiency of the post-translocation folding process determines the fate of the tyrosinase molecule. It has been reported that nascent tyrosinase chain binds calnexin co-translationally and co-translocationally, while still bound on ribosomes (15). While the soluble tyrosinase loses the post-translational calnexin interaction (10), the wild-type takes

![Figure](image)

**FIGURE 4.** WT and ST disulfide bond formation follows different kinetics. A, HEK cells were transfected with pTriEx_WT, pTriEx_ST. 24 h post-transfection, HEK293 cells were pulsed for 5 min and chased for 0, 1, and 12 min. Cell lysates were precipitated with T311 antibody and resolved by non-reducing (NR) and reducing (R) 8% SDS-PAGE and autoradiographed. The presence of aggregates is marked at the top of the gel. Red, reduced forms; ox, oxidized forms. B, same as described for panel A except that the end of the chase cells were incubated for 5 min with 5 mM DTT. Immunoprecipitated samples were run in non-reducing conditions. C, same as described for panel B except that reducing and non-reducing samples were run on the same gel. DTT-sens, DTT-sensitive form; DTT-res, DTT-resistant form. Shown is one of at least three independent experiments. WT folding pathway occurs through at least two intermediates, whereas ST folding is arrested in a DTT-sensitive conformation.

**FIGURE 5.** The TMD composition is not relevant for tyrosinase maturation and calnexin interaction. A, HEK293 cells were transiently transfected with pTriExST_Mu, pTriExST_E2. Cell lysates were divided in half; the two pools were digested (+) or not (−) with Endo H, run in reducing 10% SDS-PAGE, and subjected to immunoblotting with anti-tyrosinase antibodies. Shown is one of at least three representative experiments. B, 24 h post-transfection, the cells were prepared for anti-tyrosinase immunofluorescence. PFA-treated cells show the plasma membrane and perinuclear localization of ST_Mu and the perinuclear localization of ST_E2. C, to determine the association of ST_Mu and ST_E2 with chaperones, pulse-chased transfected cells were sequentially immunoprecipitated with antibodies to CNX and tyrosinase. The chaperone-associated tyrosinases were re-immunoprecipitated from the first immunoprecipitation complexes with anti-tyrosinase antibody. To determine the total amount of tyrosinase, the unbound proteins were precipitated from the final supernatant with anti-tyrosinase antibodies. Samples were subjected to SDS-PAGE (10%). D, levels of the chaperone associated with ST_Mu and ST_E2 (ST_Mu-CNX and ST_E2-CNX) were determined by densitometry and expressed as a percentage of the total tyrosinase value/chase point. Values indicate the mean of two independent experiments.
Transmembrane Domain Role in Ectodomain Folding

**A**

![Image](https://example.com/image.png)

**B**

![Image](https://example.com/image.png)

**C**

![Image](https://example.com/image.png)

**D**

![Image](https://example.com/image.png)

**FIGURE 6.** Addition of a GPI anchor restores the calnexin interaction and the ER exit of the tyrosinase ectodomain. HEK293 cells were transiently transfected with pTriEx ST_GPI. A, cell lysates were divided in half. The two pools were digested (+) or not (-) with Endo H, run in reducing 10% SDS-PAGE, and subjected to immunoblotting with anti-tyrosinase antibodies. Shown is one of at least two representative experiments. B, 24 h post-transfection, the cells were prepared for anti-tyrosinase immunofluorescence. Although most of the ST_GPI protein was intracellular, there was some plasma membrane localization in nonpermeabilized cells. C, to determine the association of ST_GPI with chaperones, pulse-chased transfected cells were sequentially immunoprecipitated with antibodies to CNX and tyrosinase. The chaperone-associated tyrosinases were re-immunoprecipitated from the first immunoprecipitation complexes with anti-tyrosinase antibody. To determine the total amount of tyrosinase, the unbound proteins were precipitated from the final supernatant with anti-tyrosinase antibodies. Samples were subjected to 10% SDS-PAGE. D, the extent of the calnexin-associated ST_GPI (ST_GPI-CN) was determined by densitometry and expressed as percentage of the total tyrosinase value/chase point. Values indicate the mean of two independent experiments.

Advantage of it and completes its productive folding pathway. That the presence of a TMD is a prerequisite for the regulation of cysteine oxidation by interaction with calnexin is shown for the first time here. For soluble tyrosinase, the post-translational interaction with CNX is prematurely interrupted in the absence of the TMD, yielding a misfolded protein with non-native disulfide bonds.

Because calnexin may associate also with the polypeptide (2, 3, 16), an important point was to determine whether the TMD per se is required for the calnexin/tyrosinase interaction. We found that two different TMDs restored the interaction with calnexin, indicating that there is no direct interaction between the TMD of tyrosinase and calnexin. This was also supported by the complete absence of association found in the presence of an inhibitor of the lectin-type interaction. Hence, any TMD attached to tyrosinase ectodomain determined calnexin association with the ectodomain N-glycans and consequently correct folding and secretion of the chimera. Surprisingly, the exchange of the TMD with a GPI anchor was also sufficient to restore the calnexin interaction and ER export. Taken together, the results indicate that a membrane anchor is required to bring the protein into the proximity of calnexin. Because the GPI anchor is attached to the chain after its exit from the translocon (17), we conclude that the membrane attachment favors the calnexin interaction in a step occurring post-translocationally. This supports the folding experiments showing a post-translational formation of the DTT-resistant folding intermediate of transmembrane tyrosinase.

Two possible folding scenarios may be envisaged for the folding process of the transmembrane glycoprotein ectodomains. First, the co-translational interaction with chaperones generates a folding intermediate that is committed to folding. For example, the HA folding process is strongly dependent on calnexin interaction (18, 19). HA interacts co-translationally with calnexin, and the lack of its TMD does not drastically affect the post-translational calnexin interaction (20). However, HA is able to reach the fully oxidized, DTT-resistant form following its dissociation from calnexin (21). Therefore, HA belongs to a category of proteins that require chaperone assistance only in the early stages of folding. This is consistent with a recent report showing that the HA folding pathway could be completely reconstituted in vitro, provided that the early stages of folding occurred in the intact cell (22). Second, there are polypeptides for which the co-translational chaperone assistance is insufficient to achieve a conformation committed to folding, and any perturbation in chaperone assistance during early post-translational stages could be fatal. Tyrosinase is such a protein that interacts with CNX co- and post-translocationally, with the post-translocation association being vital for folding. The TMD recruits calnexin to assist the polypeptide folding into the native conformation after its release from the translocon channel. Other proteins belonging to this category could be the viral fusion proteins of the paramyxovirus and rabies virus, which adopt different conformations in the absence of the TMD (23, 24), and the cystic fibrosis transmembrane receptor whose TMDs are released from the translocon depending on the native folding of the ectodomain (25).

We conclude that the TMD of the membrane-anchored glycoprotein tyrosinase is compulsory for its productive folding pathway. Unlike the soluble constructs, the membrane-anchored tyrosinase chimeras undertake a productive folding pathway that allows their ER export. The TMD controls tyrosinase folding pathway by regulating calnexin recruitment after the release of the newly synthesized polypeptide from the translocon channel. This is a novel role for the TMD in the folding of membrane-bound proteins that adds to our understanding of the multiple roles of TMDs and of the post-translational regulation of protein expression. Deciphering the subtle relationship between TM and the luminal domain folding is important...
because it impacts the design of expression protocols for transmembrane proteins that cannot be obtained by classical expression strategies. By monitoring the role of molecular determinants of the tyrosinase molecule, we will be able to design mutants destined to improve the immunization procedures for vaccination and serve as a defense against malignant cells.

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