Folding and Maturation of Tyrosinase-related Protein-1 Are Regulated by the Post-translational Formation of Disulfide Bonds and by N-Glycan Processing*

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In this study we have explored the endoplasmic reticulum associated events accompanying the maturation of the tyrosinase-related protein-1 (TRP-1) nascent chain synthesized in mouse melanoma cells. We show that TRP-1 folding process occurs much more rapidly than for tyrosinase, a highly homologous protein, being completed post-translationally by the formation of critical disulfide bonds. In cells pretreated with dithiothreitol (DTT), unfolded TRP-1 is retained in the endoplasmic reticulum by a prolonged interaction with calnexin and BiP before being targeted for degradation. The TRP-1 chain was able to fold into DTT-resistant conformations both in the presence or absence of α-glucosidase inhibitors, but folding occurred through different pathways. During the normal folding pathway, TRP-1 interacts with calnexin. In the presence of α-glucosidase inhibitors, the interaction with calnexin is prevented, with TRP-1 folding being assisted by BiP. In this case, the process has similar kinetics to that of untreated TRP-1 and yields a compact form insensitive to DTT as well. However, this form has different thermal denaturation properties than the native conformation. We conclude that disulfide bridge burring is crucial for the TRP-1 export. This suggests that although various folding pathways may complete this process, the native form may be acquired only through the normal unperturbed pathway.

Tyrosinase-related proteins (TRPs) include tyrosinase, TRP-1, and TRP-2. They constitute a family of membrane proteins structurally related but with distinct enzymatic functions along the biosynthetic pathway of melanin pigment in animal tissues (1). TRP-1 or glycoprotein 75 is the most abundant glycoprotein in melanocytes (2, 3) and one of the best characterized melanoma antigens (4, 5). It is well known that TRP-1 has an important role in the maturation and stability of melanosomes, site organelles of the melanin synthesis and deposition (5, 6). TRP-1 maps to the brown locus on chromosome 4 (7, 8). Unlike mutations at albino locus on chromosome 7 that encodes the tyrosinase, mutations at brown locus do not eliminate pigmentation but result in synthesis of brown instead of black eumelanin (9). Within the multimeric complex formed by TRP enzymes in melanosomal membrane (10), TRP-1 plays an important role in stabilization of tyrosinase, thus indirectly controlling the melanin production (11).

TRP-1 is a type I membrane glycoprotein with 533 amino acids, 6 potential N-glycosylation sites, 17 cysteine residues grouped in two cysteine-rich domains, and two copper binding domains (1, 12, 13). TRP-1 and tyrosinase share a significant level of homology in several regions including the cysteine-rich domains and the potential N-glycosylation sites (1).

TRP-1 from both human (5) and mouse melanoma (14) follows the regular biosynthesis pathway as most of the membrane glycoproteins. After synthesis in the ER compartment, the partially N-glycosylated polypeptide transits the Golgi compartment, and it is finally transported to its target organelle, the melanosome. The nascent polypeptide of a membrane glycoprotein is translocated into the ER compartment and folds into the native conformation assisted by molecular chaperones and other ER-resident molecules. The polypeptide compaction is associated for most of them with the formation of disulfide bonds (oxidative folding) that stabilize the native conformation. The reversible formation of disulfides is facilitated by the oxidizing environment of the ER lumen and is catalyzed by specific enzymes (15–17). In order to assess the role of disulfide formation on polypeptide intracellular transport, sorting, and processing, the ER redox potential can be manipulated by culturing the cells in the presence of reducing agents (18–22).

N-Glycan processing has been shown to be an important event involved in the folding of the nascent chain. N-Glycosylation sites are occupied in the ER compartment by the cotranslational attachment of the core glycan, Glc3Man9GlcNAc2. The trimming of Glc3Man9GlcNAc2 precursor by ER glucosidases I and II initiates the N-glycan processing and interferes with the folding events. The resulting monoglycosylated form, GlcMan9GlcNAc2, becomes substrate for the lectin chaperones calnexin and calreticulin that assist the polypeptide until the native conformation is achieved (23). In the absence of α-glucosidase I inhibitors, such as deoxyojoirimicin, N-butyl-deoxyojoirimicin (NB-DNJ), and castanospermine, the trimming of the first glucose is abolished. Thus, the glucosylated N-glycans cannot be further processed, and glycoprotein folding cannot be controlled by the calnexin cycle. Calnexin and calreticulin have been shown to direct the bound polypeptide chain into deglycosylation/reglycosylation cycles in which the lectins...
act in conjunction with glucosyltransferase and α-glucosidase II to retain the nascent chain in repeated binding and release cycles until complete folding (24). The components of the calnexin cycle are elements of the ER quality control system, which eliminates the grossly misfolded proteins and allows the export of the correctly folded ones. It has been reported that another component of the quality control is the chaperone BiP, the immunoglobulin heavy chain binding protein. BiP is a molecular chaperone that transiently interacts with unfolded stretches of a nascent polypeptide as long as these regions have not reached their folded conformation (25). Some proteins remain bound to BiP until they are degraded, and a role for BiP as a lid of the translocon pore has been recently suggested (26). Although sequential interactions of the chain with the two chaperones and complexes between calnexin and BiP have been reported, it is still unclear if BiP and calnexin are required for the correct folding of any individual glycoprotein (27).

We have previously shown that inhibition of early N-glycan processing with NB-DNJ resulted in tyrosinase inactivation and a dramatic loss of cell pigmentation in melanoma cells (28). It has been also shown that the inhibitory effect of NB-DNJ affected dramatically the folding pathway of the nascent chain. This proved to be strictly controlled by the interaction with calnexin (29–31). Our more recent studies demonstrated that under the same inhibitory conditions in the same cell line, the TRP-1 polypeptide chain was able to overcome the glucosidase blockade by the action of the Golgi endomannosidase, which allowed the further processing of the N-glycans to complex structures (32). These findings prompted us to hypothesize that in the presence of the ER glucosidase inhibitors TRP-1 chain could fold by alternative pathways to a different conformation that could also influence the further processing of its N-glycans (for a review see Ref. 33).

In this paper, we investigated the folding pathway of TRP-1 synthesized in mouse melanoma cells. We demonstrate that disulfide bonds post-translationally formed are essential for TRP-1 maturation and stability. The nascent chain is retained in the ER until the attainment of a conformation with a disulfide bond pattern conferring DTT resistance. During folding, TRP-1 interacts with calnexin, and this interaction is prolonged when the S–S bonds are prevented from forming. The inhibition of N-glycan processing in the ER perturbs but does not completely prevent TRP-1 folding and maturation and does not affect its stability. In NB-DNJ-treated cells TRP-1 polypeptide, which does not bind to calnexin but binds to BiP, acquires a conformation stabilized also by disulfides.

MATERIALS AND METHODS

Reagents, Antibodies, and Enzymes—NB-DNJ was a gift from Searle Monsanto (St. Louis, MO). N-Ethylmaleimide (NEM), diithityritol (DTT), HEPS, CHAPS, l-methionine, and apyrase were from Sigma. Protein A-Sepharose was from Amersham Pharmacia Biotech and protease mixture inhibitor (CompleteTM) from Roche Molecular Biochemicals. The rabbit anti-TRP-1 antiserum (anti-TRP-1) was purchased from the Laboratory of Dr. Corrin Gray (University of Florida, Gainesville, FL). Endo H F and PNGaseF were from New England Biolabs (Beverly, MA). All other chemicals were from Sigma. Cell Culture—B16 F1 mouse melanoma cells (European Collection of Animal Cell Cultures, Porton Down, UK) were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing 10% (v/v) fetal calf serum (Serum), 50 units/ml penicillin, and 50 mg/ml streptomycin (Life Technologies, Inc.). The cells were maintained at 37 °C in an atmosphere of air/CO₂ (19:1).

Pulse-Chase Experiments—B16 mouse melanoma cells were harvested with EDTA washed three times with 0.1 M phosphate-buffered saline, pH 7.2, and resuspended in methionine- and cysteine-free RPMI 1640 medium (Life Technologies, Inc.). Cells (10⁵ cells/ml) were preincubated for 1 h at 37 °C before the addition of [35S]methionine/[35S]cysteine at 200 μCi/ml. Following the labeling period of 10 min, RPMI medium containing 5 mM unlabeled methionine and 5 mM DTT was added to the cells 5 min before the pulse and maintained at the same concentration during the chase periods. In NB-DNJ experiments cells were cultured before labeling for 2 h in normal medium containing 5 mM NB-DNJ, and the inhibitor was further maintained at the same concentration during the chase periods. At the indicated times, the chase media were removed, and the cells were harvested by scraping into cold phosphate-buffered saline. Samples analyzed for S–S bond formation were incubated before lysis for 1 h at 4 °C with 20 mM NEM to block the free SH groups and to prevent the nonspecific formation of S–S bonds. Cells were lysed in 0.5 ml of lysis buffer (50 mM HEPES, pH 7.5, containing 2% (w/v) CHAPS and 200 mM NaCl, and proteinase inhibitors), for 1 h on ice. When samples were to be used for immunoprecipitation of BiP, cells were lysed in the presence of 20 units/ml apyrase (to enzymatically deplete ATP).

Immunoprecipitation and SDS-PAGE—[35S]-Labeled cell lysates were preclotted with 20 μl of protein A-Sepharose for 2 h at 4 °C and incubated with aPEP1, anti-calnexin, or anti-BiP antiserum for 2 h at 4 °C. The immunocomplexes were separated by incubation with 20 μl of protein A-Sepharose for 2 h at 4 °C. The slurry was washed with 50 mM HEPES, pH 7.5, containing 0.5% CHAPS and 200 mM NaCl. TRP-1 was further eluted in 1% SDS for 1 h at room temperature divided in three (a) mixed before running with SDS-PAGE sample buffer without β-mercaptoethanol (non-denaturing and non-reducing conditions), (b) incubated for 5 min at 100 °C and mixed with SDS-PAGE sample buffer without β-mercaptoethanol (denaturing and non-reducing conditions), (c) incubated in SDS-PAGE sample buffer with β-mercaptoethanol for 5 min at 100 °C (denaturing and reducing conditions). When TRP-1 bound to calnexin or BiP was analyzed, samples were eluted for 1 h at room temperature in lysis buffer containing 1% SDS. SDS concentration was decreased in eluates at 0.1%, and TRP-1 was immunoprecipitated with aPEP1. Unless other specifications were made, samples were run in SDS-7.5% PAGE and analyzed by autoradiography.

Enzyme Digestion—[35S]-Labeled samples were digested with Endo H or PNGaseF as described (14). Briefly, TRP-1 samples were eluted from the protein A-Sepharose in Endo H or PNGaseF denaturing buffer, by incubation for 5 min at 100 °C. The eluted amount was digested in the reaction buffer of either Endo H or PNGaseF with 500 units of Endo H or PNGaseF for 18 h at 37 °C, run in SDS-PAGE, and analyzed by autoradiography.

RESULTS

TRP-1 Folding in B16 Mouse Melanoma Cells—We monitored TRP-1 folding in pulse-labeled B16 cells by immunoprecipitation of cell lysates at different chase time points with aPEP1 antiserum (34) and analysis of TRP-1 by non-reducing SDS-PAGE. In non-reducing SDS-PAGE, a protein in an open or reduced conformation migrates slower than its compact or oxidized form. As shown in Fig. 1A, the slower migrating bands observed at 0 and 15 min of chase (lanes 1 and 2) are replaced by a faster migrating band at 30 min (lane 3). This indicates that the TRP-1 chain collapses to a compact form in 30 min after synthesis. No further increase in mobility at 45, 60, or 120 min of chase was detected (lanes 4–6) showing that further processing to complex type N-glycans has no effect on the migration of TRP-1. A similar behavior was observed for tyrosinase in non-reducing gels (29). Our interpretation is that in non-reducing gels, the migration velocity of TRP-1 is dominated by the mass rather than by the molecular weight, which in turn is prevailing in reducing gels. To characterize the folding kinetics of the TRP-1 chain, the above samples were analyzed in SDS-PAGE under (a) non-denaturing and non-reducing conditions, (b) denaturing and non-reducing conditions, and (c) denaturing and reducing conditions (see “Materials and Methods”). As can be observed in Fig. 1B a faster migration was detected for the non-denatured (a) versus denatured (b) samples at 15, 30, and 45 min (lanes 4 and 5, 7 and 8, and 10 and 11). However, no differences in the TRP-1 sample...
FIG. 1. TRP-1 folding in B16 mouse melanoma cells. A. B16 cells were pulsed for 10 min with $^{35}$S and chased for the indicated periods. Cell lysates were immunoprecipitated with αPEP1 antiserum, and the eluted samples were run in 10% SDS-PAGE under non-reducing conditions. B, each sample at 0, 15, 30, and 45 min of chase was run in 7.5% SDS-PAGE in non-denaturing and non-reducing conditions (a), denaturing and non-reducing conditions (b), denaturing and reducing conditions (c) as described under “Materials and Methods” (B). All samples were visualized by autoradiography.

migration at 0 min of chase before and after thermal denaturation can be detected (lanes 1 and 2). This indicates that some secondary structures are gradually formed in TRP-1 during folding. Moreover, the reduced forms of TRP-1 (c) migrate significantly higher than the non-reduced denatured ones (b) at all chase points, and this proves that TRP-1 carries disulfides.

Disulfide Bond Formation during TRP-1 Folding—To determine whether the S—S bonds are involved in TRP-1 folding, we analyzed the folding process in the presence of the reducing agent DTT. This agent can quickly penetrate across the cell membrane and prevents the formation of disulfides in nascent proteins (20, 35). Cells were incubated 5 min before pulse, pulse-labeled, and chased in the presence of 5 mM NEM to block the free SH groups and to prevent the nonspecific formation of S—S bonds. TRP-1 was immunoprecipitated from untreated and DTT-treated cell lysates, and samples were analyzed in non-reducing conditions in SDS-PAGE (Fig. 2). In all DTT-treated samples, TRP-1 migrates as a band with constant mobility representing TRP-1 in its fully reduced conformation (lanes 2, 4, 6, and 8). In the absence of DTT, TRP-1 at 0 min chase (lane 1) is already in a partially folded conformation demonstrated by its slightly increased mobility, when compared with the TRP-1 DTT-treated (lane 2). The difference between the DTT-treated and untreated samples at 15 min chase (lanes 3 and 4) is even more pronounced indicating that TRP-1 chain becomes more compact within this period. It can be estimated that 30 min post-pulse TRP-1 has attained the fully oxidized conformation, as the difference in the electrophoretic mobility between DTT-treated and untreated samples at 30 and 45 min chase is identical (lanes 5 and 6 and 7 and 8). Since in the presence of DTT, the appearance of the fully oxidized chain is prevented, it can be assumed that the two folding intermediates observed at 0 and 30 min (Fig. 1A, lanes 1 and 3 and Fig. 2, lanes 1 and 5) are different disulfide intermediates.

To discriminate between the different folding stages of TRP-1, an experiment was designed to block the formation of disulfide bridges at various stages during TRP-1 folding followed by the monitoring of the maturation pathway of the intermediates of folding (Fig. 3). Cells were pulse-labeled for 10 min and chased for 0, 15, 30, and 45 min in normal medium before the addition of DTT to each sample. The chase was continued for another 45 min in the presence of the reducing agent; hence, the total chase time in this experiment was 45, 60, 75, and 90 min, respectively. At the end of the chase time cell lysates were immunoprecipitated with αPEP1 antiserum and analyzed in non-denaturing and non-reducing SDS-PAGE (see “Materials and Methods”) and visualized by autoradiography.

Fig. 2. Kinetics of S—S bond formation in TRP-1 in B16 mouse melanoma cells. B16 cells were pulsed for 10 min with $^{35}$S and chased for the indicated periods. In DTT-treated cells, the reducing agent was added 5 min before pulse in a concentration of 5 mM, and the cells were chased in the continuous presence of the reducing agent. Cells were incubated before lysis with 20 mM NEM, and TRP-1 from both untreated (−) and DTT-treated (+) cells was immunoprecipitated with αPEP1 antiserum, and samples were analyzed in non-denaturing and non-reducing SDS-PAGE (see “Materials and Methods”) and visualized by autoradiography.
the presence of DTT in the culture medium.

**TRP-1 Maturation and Transport Depend on the Formation of S—S Bonds**—To investigate the role of disulfides in TRP-1 maturation, the samples from the previous pulse-chase experiment (presented in Fig. 2) were analyzed under reducing conditions (Fig. 4A). TRP-1 chain from untreated cells migrates at 69 kDa for the first 15 min (lanes 1 and 3) showing an increase in molecular mass to 75 kDa at 45 min of chase (lane 5). In DTT-treated cells, TRP-1 migrated as a 69-kDa precursor for all chase times analyzed (lanes 2, 4, and 6) suggesting that no maturation occurred in the presence of DTT. Our previous studies have shown that within 1 h of chase TRP-1 acquires complex oligosaccharides becoming partially resistant to Endo H digestion (14). Indeed, after Endo H treatment, which completely removes high mannose and hybrid oligosaccharides attached to the polypeptide, TRP-1 from DTT-treated sample at 1 h of chase co-migrated with untreated TRP-1 at 0 min of chase (Fig. 4A, lanes 7 and 8). These results indicate that the glycans attached on TRP-1 synthesized in the continuous presence of DTT were not further processed to complex structures.

To monitor the degradation process of TRP-1 synthesized in the presence of DTT, TRP-1 samples immunoprecipitated from B16 cells pulse-chased for the time points shown in Fig. 4B were analyzed by SDS-PAGE under reducing conditions. There was a significant decrease in the intensity of the TRP-1 band from 0 to 1 h of chase (lanes 1 and 2) followed by a gradual disappearance of the band. In contrast, TRP-1 in the untreated cells was shown to have a half-life longer than 5 h (data not shown) indicating that TRP-1 degradation is accelerated when the glycoprotein is synthesized in the continuous presence of DTT.

**TRP-1 Folding in the Presence of ER Glucosidase Inhibitors**—TRP-1 is a glycoprotein with 6 potential N-glycosylation sites. To determine whether N-glycosylation has a role in TRP-1 folding, we monitored TRP-1 folding in the presence of N-butyl-deoxynojirimicin (NB-DNJ). NB-DNJ is an inhibitor of α-glucosidases I and II, which prevents N-glycan trimming in ER compartment and calnexin binding to the monoglucosylated protein precursors (28). Two hours before pulse the inhibitor was added to the culture medium to a concentration of 5 mM and maintained during the pulse-chase process. The cells were pulsed for 10 min with [35S]methionine and chased for 0, 15, and 60 min. TRP-1 immunoprecipitated from the cell lysates was analyzed in SDS-PAGE under (a) non-denaturing and non-reducing conditions, (b) denaturing and non-reducing conditions, and (c) denaturing and reducing conditions as described under “Materials and Methods.” As shown in Fig. 5A the native (a) and denatured non-reduced (b) samples co-migrate in SDS-PAGE for all chase time points. Unlike TRP-1 normally processed, TRP-1 from NB-DNJ-treated cells does not change its conformation after thermal denaturation. The reduced form of TRP-1 (c) from NB-DNJ-treated cells migrated in a slightly higher position than the non-reduced forms, and this served as a proof for the presence of disulfide bonds. It could be concluded that the TRP-1 polypeptide chain was able to fold into an oxidized form despite the presence of the N-glycosylation inhibitor in the culture medium.

Since the folding rate of tyrosinase is considerably accelerated in the presence of NB-DNJ (29), it was of interest to determine the kinetics of folding to a stable conformation of the TRP-1 chain under similar conditions. Cells were incubated for 2 h in the presence of 5 mM NB-DNJ, pulsed for 10 min, and chased in the presence of the inhibitor for 0, 15, 30, and 45 min. After chase, 5 mM DTT was added to each sample, and the chase was continued in the presence of both NB-DNJ and DTT. For better evidence of the TRP maturation along the indicated chase points, samples were digested with Endo H (Fig. 5B, lanes 1–4). A sample of mature TRP-1, synthesized in the presence of NB-DNJ only, was completely deglycosylated with PNGase F thus indicating the position of the TRP-1 polypeptide (lane 5). The results of this experiment show that when TRP-1 is synthesized in the presence of NB-DNJ up to 15 min post-pulse and another 45 min with both NB-DNJ and DTT, the protein remained totally sensitive to Endo H (lanes 1 and 2). When DTT is added after 30 and 45 min and maintained for another 45 min, TRP-1 synthesized in the presence of the inhibitor becomes more resistant to Endo H and acquires oligosaccharides of complex structure (lanes 3 and 4). The Endo H digestion pattern of the last two chase points is similar with the pattern of mature NB-DNJ TRP-1 reported previously (14). The results indicate that TRP-1 synthesized in the presence of NB-DNJ acquires the competent transport conformation, being resistant to further DTT exposure, within approximately the same time as TRP-1 from untreated cells.

**TRP-1 Interaction with ER Resident Chaperones**—By having established that inhibition of glucose trimming influences TRP-1 folding, investigations were performed to determine the role of the chaperone calnexin in TRP-1 folding. B16 cells were pulse-labeled for 10 min with [35S]methionine and chased for 0, 15, 30, and 60 min. Cells were lysed in HEPES/CHAPS buffer that preserves the interactions of calnexin with substrate proteins (36) and immunoprecipitated sequentially with anti-calnexin antiserum followed by aPEP1 antisera. The results presented in Fig. 6 (lanes 1–5) showed that TRP-1 bound to calnexin was detected immediately after pulse, and the amount of TRP-1 bound to calnexin decreased progressively after 15 and 30 min of chase. After approximately 45 min of chase no interaction with calnexin was detected. This was expected since, as we previously reported, at 45 min of chase TRP-1 appeared as a fully glycosylated protein that has already passed the ER compartment (14). It can be observed that the time course of TRP-1 associated with calnexin is well correlated with the formation of the fully oxidized chain in approximately 30 min post-pulse. Next, we investigated the interaction of TRP-1 with calnexin under conditions where S—S bonds in TRP-1 are prevented from forming. The cells were incubated 5 min before pulse with 5 mM DTT, pulse-labeled for 10 min, chased for 45 min and 1 and 3 h in the presence of 5 mM DTT, and TRP-1 was...
Compared with the untreated cells, when after 30 min we could not detect any interaction with calnexin (Fig. 6, lanes 4 and 5), in DTT-treated cells TRP-1 is bound to calnexin for up to 1 h of chase (lane 6). Although we did not detect any interaction with calnexin in NB-DNJ-treated cells (data not shown), we hypothesized that another chaperone could assist TRP-1 during folding to acquire its stable conformation. Analysis of TRP-1 association with the ER-resident chaperone BiP was performed in B16 cells pulsed 10 min and analyzed for 5, 10, and 45 min (Fig. 7) in the presence or absence of NB-DNJ and DTT. Cell lysates were immunoprecipitated with aPEP1 antiserum and analyzed in reducing SDS-PAGE. We observed that significantly more TRP-1 is co-immunoprecipitated by BiP in NB-DNJ-treated cells than in the untreated controls for the same chase period (lanes 1–3 and 4–6). TRP-1 also binds to BiP up to 45 min chase (lanes 7–9) in DTT-treated cells, in the absence of disulfides. The amount of TRP-1 bound to BiP slightly decreased within 10 min of chase in NB-DNJ-treated cells (lanes 4–6), and no interaction was detected at 30 min of chase (data not shown).

**DISCUSSION**

TRP-1 is a melanosomal membrane glycoprotein that is synthesized in the ER, transits the Golgi apparatus, and accumulates in the trans-Golgi network before being targeted to the melanosome. We have recently shown that in B16 mouse melanoma cells cultured in the presence of NB-DNJ, TRP-1 transport, maturation, and degradation depend on the disulfide bonds formation. A, B16 cells were labeled for 10 min with 35S and chased in the absence (−) and presence (+) of 5 mM DTT for the indicated periods. TRP-1 was immunoprecipitated from cell lysates with aPEP1 antiserum, and samples were run in reducing SDS-PAGE (lanes 1–6). To observe the maturation of complex oligosaccharides, TRP-1 from untreated cells at 0 min of chase (lane 7) was compared with TRP-1 at 60 min of chase from DTT-treated cells digested with Endo H (lane 8). Samples were visualized by autoradiography. B, cells were labeled for 10 min with 35S and chased in the presence of 5 mM DTT up to 4 h, analyzed in reducing SDS-PAGE, and visualized by autoradiography.
anoma cells TRP-1 is synthesized as a precursor polypeptide of 69 kDa acquiring the first complex oligosaccharide structures in the Golgi in approximately 30 min of chase (14). Within approximately 45 min of chase the TRP-1 polypeptide chain is completely processed to a 75-kDa mature glycoprotein (14). A similar time course of maturation has been reported for TRP-1 synthesized in human melanoma cells (5) documenting the same ER residence time for this glycoprotein in two different melanoma cells in vivo.

In this study, we have investigated the ER-associated events accompanying the folding of the nascent TRP-1 polypeptide chain required for further trafficking through the secretory pathway. We show here that TRP-1 synthesized in mouse melanoma cells folds into the most compacted conformation in the ER in less than 30 min post-pulse. The final three-dimensional conformation appears to be stabilized not only by native disulfide bridges but also by non-covalent interactions sensitive to thermal denaturation, as shown by the SDS-PAGE analysis of the pulse-chase immunoprecipitates. During the folding process, which is completed post-translationally, two folding intermediates with different electrophoretic mobilities in non-reducing gels have been detected. They differ in their disulfide bonding patterns as shown by the pulse-chase experiments performed in the presence of the reducing agent DTT.

The time course formation of stable intermediates during the TRP-1 folding process was monitored by a post-pulse DTT treatment experiment. This approach allowed the proper folding of the chain before the DTT exposure occurred but prevented any further disulfide bond formation, thus facilitating the identification of stable DTT-resistant conformers. We observed that TRP-1 polypeptide chain does not fold immediately after synthesis in a stable conformation. This is gradually completed by 30 min of post-pulse, a time point coinciding with the appearance of the most oxidized TRP-1 intermediate, followed by its export from the ER. Although the co-translational formation of some disulfides cannot be ruled out, our data support the notion that native disulfides are gradually formed post-translationally, being required for the completion of the oxidative folding process.

The importance of post-translational disulfide bonds in the maintenance of the native conformation is different from one protein to another. In hemagglutinin-neuraminidase protein from Newcastle disease virus, the protein forced to form all disulfides post-translationally has no biological activity (38). The folding pathway and the final conformation of the protein were altered, despite the collapse of the chain to a compact conformation stabilized by disulfide bridges. Similarly, the β-subunit precursors of the human chorionic gonadotropin, despite incorrect folding (39), could collapse to a conformation competent for dimerization with the α-subunit in presence of DTT (40). On the contrary, disulfide bond formation in the acetylcholine receptor occurs post-translationally, preceding the chain conformational maturation and subunit assembly (41). Our pulse-chase experiments in the continuous presence of DTT followed by the immunoprecipitation of TRP-1 showed that disulfide bond formation is crucial for the transport, maturation, and degradation processes of the nascent chain. By blocking the S—S bonds formation, the reducing agent also prevented TRP-1 oxidative folding affecting the protein maturation process. As a consequence, TRP-1-synthesized in the continuous presence of DTT does not reach the trans-Golgi compartment and therefore does not acquire complex oligosaccharides being targeted to degradation much faster than the normally processed chain. It is very likely that TRP-1 in its fully reduced conformation cannot escape the ER quality control system and is probably eliminated by a common mechanism adopted for other severely misfolded proteins (42). Knowing that besides TRP-1, all the other members of the TRP family contain 15 cysteine residues in well conserved positions along the polypeptide chain, one could consider the role of disulfide bridges for the biological function of TRPs (1, 12, 13). However, there is no clear information at this time on the number and location of the S—S bonds on these chains. Interestingly, two classic mutations, in tyrosinase (albino) and in TRP-1 (brown), are substitutions of Cys-5 and Cys-4, respectively, both localized within the epidermal growth factor domain, with a well characterized pattern of the S—S bonds in many proteins (43). A more recently characterized human al-

![Fig. 6. Interaction of TRP-1 with calnexin is prolonged in DTT-treated cells. B16 cells were pulsed for 10 min with 35S and chased in the absence (−) up to 60 min (lanes 1–5) or in the presence of 5 mM DTT (+) for 180 min (lanes 6–8). TRP-1 bound to calnexin was retrieved by double immunoprecipitation with anti-calnexin and αPEP1 antisera and run in reducing SDS-PAGE. Samples were visualized by autoradiography.](image1)

![Fig. 7. TRP-1 interaction with BiP in NB-DNJ- and DTT-treated cells. B16 cells were pulsed for 10 min with 35S and chased in the absence (−) and presence (+) of 5 mM NB-DNJ for 0, 5, and 10 min or in the presence of 5 mM DTT (+) for 0, 10, and 45 min. TRP-1 bound to BiP was retrieved by double immunoprecipitation with anti-BiP and αPEP1 antisera, run in reducing SDS-PAGE, and detected by autoradiography.](image2)
binism mutation is the substitution of Cys-2 with Arg in tyrosinase (44). Although the mechanism of tyrosinase and TRP-1 inactivation in these genetic disorders is not well characterized, it is believed that tyrosinase-negative albinism is an ER retention disease (45, 46). As the ER retention is a process associated with the misfolding and degradation of the nascent chain, these observations together with our data could imply that critical cysteine residues may be involved in designing the native conformation of TRP polypeptide chain. Further studies are needed for a better understanding of the role of particular cysteines in the formation of the correctly folded conformation of TRP glycoproteins.

The structural homology between TRP-1 and tyrosinase is not restricted only to the position of the cysteine residues, as it has been already mentioned. Another important similarity in their structure is the presence of six potential glycosylation sites on the polypeptide chain (1, 12, 13). In the case of tyrosinase we have previously shown that correct folding of the chain is highly dependent on the association of the folding polypeptide with the ER resident chaperones calnexin/calreticulin (29). Moreover, the kinetics of the folding pathway is changed when the interaction with calnexin/calreticulin is prevented. In the presence of the α-glucosidases inhibitor NB-DNJ, the tyrosinase chain folds “faster” to an inactive state (29). Surprisingly, the data presented here show that TRP-1 synthesized in B16 melanoma cells treated with NB-DNJ folds with similar kinetics as under normal conditions. In the presence of this drug, TRP-1 polypeptide is able to fold to an oxidized form stabilized by disulfide bridges, as shown by the electrophoretic pattern of the chain in reducing versus non-reducing conditions. Moreover, the post-pulse DTT experiment revealed that in the presence of NB-DNJ a stable conformation preserved by S—S bonds is acquired within 30 min post-pulse as is observed in the normally processed protein. However, the conformation of this chain is different from the conformation of normal TRP-1 as suggested by the different behavior toward thermal denaturation. These results confirm previously reported data showing that NB-DNJ-treated TRP-1 had a different conformation (32) and acquired less complex oligosaccharides (14) as compared with the normally processed protein.

The co-immunoprecipitation experiments with anti-calnexin and αPEP1 antiserum showed that TRP-1 associates with calnexin for the first 30 min of synthesis until the chain acquires its native conformation. Under conditions that maintain the polypeptide in a reduced but glucosylated state, such as in DTT treatment, TRP-1 shows a prolonged interaction with calnexin before being targeted to degradation. In contrast to TRP-1 synthesized in normal conditions, NB-DNJ-treated TRP-1 does not interact with calnexin but with BiP. In NB-DNJ-treated cells, TRP-1 binds avidly to BiP, whereas in normally processed NB-DNJ-treated TRP-1 this interaction is hardly detected. It is known that BiP binds to the hydrophobic patches that are transiently exposed mainly during the early steps of polypeptide folding, and this was confirmed by the prolonged interaction of BiP with TRP-1 in DTT-treated cells. The poor interaction of TRP-1 with BiP in the untreated cells indicates that TRP-1 hydrophobic patches are rapidly folded into a conformation with very little accessibility to BiP. In contrast, interaction with BiP is dramatically increased in the absence of calnexin and prolonged in the presence of denaturing agents. Interestingly, dissociation of the chain from BiP coincides with the appearance of the oxidized form of NB-DNJ-treated TRP-1 and with the targeting for degradation moment of TRP-1 synthesized in the presence of DTT. Collectively, these results indicate that in the folding process of TRP-1, BiP is used in the absence of the lectin chaperone calnexin and that under severe stress BiP and calnexin act simultaneously to help the polypeptide chain folding. Further confirmation comes from previously reported experiments showing that BiP associates especially well with early folding intermediates and can serve as a backup for calnexin or calreticulin in retaining partially folded structures (47, 48).

In conclusion, the maturation and stability of TRP-1 polypeptide chain are differently affected by perturbation of the folding process. Under normal conditions in vivo, the TRP-1 chain adopts a conformation stabilized by intramolecular S—S bonds and non-covalent interactions. As in the case of the highly homologous tyrosinase, TRP-1 folding is completed post-translationally. In contrast to tyrosinase, the time course of TRP-1 folding in melanoma cells is 6-fold faster. We were able to demonstrate that the time spent by TRP-1 in the ER is required for the attainment of the stable compact conformation resistant to DTT attack. Similar to tyrosinase, the TRP-1 nascent chain folding is mediated by calnexin. In case of inhibition of N-glycan processing in the ER, the interaction with calnexin is prevented. In contrast to NB-DNJ-treated tyrosinase that folds faster than the wild type, NB-DNJ-treated TRP-1 assisted by BiP is able to fold with similar kinetics as the untreated TRP-1 and acquire the DTT-resistant conformation. However, this conformation is different from the one of wild type, and it may influence the protein processing in the next compartments of the secretory pathway (14). When the protein is forced to adopt a fully reduced conformation, its folding and maturation are prevented, the polypeptide chain being retained in the ER by prolonged association with the chaperones calnexin and BiP, prior of being targeted to degradation.

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