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Protein Disulfide Isomerase Acts as a Molecular Chaperone in the Intracellular Retention of Mouse Mutant Thyroglobulin

Piyanuch Jongsamak*

Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

* Corresponding author, E-mail: jongsamak@yahoo.com

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ABSTRACT: Relatively few point mutations in the thyroglobulin (Tg) gene that cause thyroid diseases have been identified in man. Here, we have examined the intracellular fate of a mouse full-length missense Tg mutant (R39K) that is equivalent to the corresponding human Tg mutant recently reported in a Brazilian kindred with congenital goiter and hypothyroidism. When expressed in COS-7 cells, markedly reduced export of the R39K Tg was associated with increased stable association with the endoplasmic reticulum (ER) chaperone BiP/GRP78, pointing to a folding defect. More prolonged association with calnexin was also observed, suggesting an important role for the lectin pathway of ER quality control in the processing of the mutant Tg that had not been previously described. Moreover, the most stable chaperone-Tg association was observed for protein disulfide isomerase (PDI), which normally functions as a redox foldase, providing new evidence that PDI may also be a molecular chaperone in the intracellular processing of the mutant Tg. Eventually, R39K Tg was degraded by the 26S proteasome in the cytosol. It was concluded that these three ER chaperones, BiP/GRP78, calnexin, and PDI are part of a quality control machinery that associates with the mutant Tg, as it is targeted for ER-associated degradation.

KEYWORDS: mutant thyroglobulin, molecular chaperones, protein folding, defective export.

INTRODUCTION

Throughout the world, it has been estimated that primary congenital hypothyroidism affects 1 in 3,000 to 1 in 4,000 newborns. About 80% of the cases are caused by the abnormal development of the thyroid gland while the remaining 20% are associated with defects in the thyroid hormone biosynthetic pathway, often characterized by the presence of goiters of varying sizes. In the latter, responsible mutations in the thyroglobulin (Tg), thyroid peroxidase (TPO), pendrin (PDS), and sodium-iodide symporter (NIS) genes have been well established. Of these, a defective thyroglobulin (Tg) molecule appears to be one of the major causes of thyroid dyshormonogenesis, affecting 1 in 40,000 newborns[1].

Located on the long arm of chromosome 8q24, the human Tg gene contains 48 exons and transcribes an mRNA that is 8.7 kb in length containing an open reading frame (ORF) of 8307 nucleotides[2]. The Tg cDNA was first cloned and sequenced in 1987[3], but it was not until 1991, when the first Tg mutation causing deletion of exon 4 was found to be associated with abnormal expression of the Tg protein[4]. Since then, two additional in-frame deletion mutants, a 171 nt del[5] and a 138 nt del[6], have been reported in several offspring of consanguineous marriages. More recently, several full-length missense mutations have been reported in kindred from Spain (Q851H)[7], Japan (C1263R[8], C1995S[8], V599E[9] and K600E[9]), Brazil (R39K[10] and A2234N[11]), and the Netherlands (G58S)[12]. Of these, limited biochemical analyses using frozen thyroid tissues obtained following surgical thyroidectomies are available only for the C1263R and C1995S mutant Tg. The other Tg mutations have been strongly suggested by linkage analysis or from complete sequencing of the Tg cDNA. However, the presence of significant polymorphisms has made it difficult to confirm the precise responsible mutations in the remaining cases.

Vital to vertebrate survival, Tg is a large secretory protein that provides the matrix for both iodide storage and thyroid hormone synthesis. Normal Tg is synthesized and cotranslationally translocated to the rough endoplasmic reticulum (ER) where it undergoes N-linked glycosylation and sugar trimming. The latter appears to be important for generating sugar signals which are recognized by the ER quality control (QC) machinery that ensures the fidelity of folding and export of nascent chains[13]. Mature Tg is a 660 kD homo-dimer containing 10% of its weight as carbohydrates and 244 cysteines that form approximately 120 disulfide

bonds[14]. Both disulfide oxidation and correction of mispaired disulfides that occur during protein folding are relatively slow steps in folding and, thus, are thought to be catalyzed by an ER resident folding enzyme known as protein disulfide isomerase (PDI)[15]. Importantly, the slow and complex Tg folding process is thought to be assisted by multiple ER molecular chaperones, which transiently bind to the nascent Tg.

Classically, chaperones are highly conserved, ubiquitous proteins that transiently bind to unstable hydrophobic regions of the nascent chains until these regions can be buried in the interior of the molecule as it folds. This family of proteins includes BiP/GRP78 and GRP94[16]. In addition, the ER uniquely also contains lectin chaperones, calnexin and calreticulin, which are able to bind to sugar and/or peptide regions of the nascent glycoproteins that have not achieved their proper tertiary structures[17]. Unlike other intracellular compartments, ER environment is relatively oxidizing, thereby favoring the formation of disulfide bonds[18]. This necessitates the presence of one or more oxidoreductases, ERp72, ERp57, and protein disulfide isomerase, which serve not only to accelerate disulfide formation, but also to provide oxidizing equivalents and catalyze disulfide rearrangements[15]. Recently, other major functional roles such as stabilizing, unfolding, and perhaps targeting misfolded wild type or mutant proteins for intracellular degradation by the 26S proteasome have been suggested for both ER chaperones and oxidoreductases[19]. Together, these and other ER resident proteins make up the quality control machinery that determines whether a protein is targeted for ER retention, export from the ER to the Golgi, or degradation.

For many years, it was thought that the ER resident proteins also include ER proteases that degrade retained misfolded proteins inside the ER by a process that was referred to as 'ER degradation'[20]. This nonlysosomal mechanism, later renamed 'ER-associated degradation' (ERAD), was judged to be essential in preventing toxic accumulation of misfolded proteins in this folding compartment. However, despite much effort, isolation of such an ER-protease has eluded investigators for the most part. This is not surprising in light of recent evidence suggesting that the actual proteolytic process appears to occur outside the ER by the proteasome-ubiquitin pathway located in the cytosol[21,22,23]. Later reports further suggested that misfolded proteins may be transported back to the cytosol from the ER through the same protein channel that is used in the co-translational translocation of nascent polypeptides into the ER[24,25,26].

Here, we have confirmed using a mouse model that the R39K mutant Tg that was recently reported in a Brazilian kindred with congenital hypothyroid goiter[10] is defective for export and is retained in the ER. Eventually, R39K Tg is degraded by the 26S proteasome in the cytosol. Moreover, we found that three ER chaperones, BiP/GRP78, calnexin, and PDI participate in the ER-associated degradation of R39K Tg.

Materials and Methods

The supplies of chemical reagents used were as follows: MG-132 (Calbiochem, La Jolla, CA), QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), Easytag™ express protein labeling-mix [^{35}S] (Perkin Elmer, Boston, MA), endoglycosidase-H (New England Biolabs, Beverly, MA), Zysorbin A beads (Zymed Laboratories, San Francisco, CA), LipofectAMINE reagent (Invitrogen, Carlsbad, CA), ECL, Hybond ECL Nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), Autoradiography Film (Labscientific Livingston, NJ), FBS, penicillin, and streptomycin (Life Technologies, Rockville, MD), anti-rabbit immunoglobulin peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA), aprotinin, apyrase, DFP, leupeptin, pepstatin A, and PMSF (Sigma, St. Louis, MO). Polyclonal rabbit antisera's to BiP, calnexin, and PDI were raised against C-terminal peptides of the respective proteins. Antibodies to thyroglobulin were raised in rabbits against the whole mouse Tg molecule.

Site-Directed Mutagenesis of Mouse Tg cDNA

A QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make the desired missense mutation in the full-length normal mouse Tg cDNA[27] in the pcDNA3.1(-)myc-his vector (Stratagene) according to the manufacturer's instructions. The sense primer T1 (5'-CCC TGT GAG CTA CAA AAA GAG AAA GCC TTT CTG-3') and the anti-sense primer T2 (5'-CAG AAA GGC TTT CTC TTT TTG TAG CTC ACA GGG-3') were used to change an arginine to lysine at residue 39. The sense primer T3 (5'-GTG TTG GAG GGA GCC CAA ACT CCG CCT GGG-3') and the anti-sense primer T4 (5'-CCC AGG CGG AGT TTG GGC TCC CTC CAA CAC-3') were used to change an arginine to glutamine at residue 855. The sense primer T5 (5'-CCA AGC TCC TGG CTC TGA GTG GCC CTT TCC-3') and the anti-sense primer T6 (5'-GGA AAG GGC CAC TCA GAG CCA GGA GCT TGG-3') were used to change a valine to leucine at residue 2472. The plasmid products were transformed into *E. coli*, which were grown on ampicillin-containing LB agar plates. Colonies were selected and screened for the desired mutation by sequencing.

Cell Culture and Transient Transfection of Mutant Tg

COS-7 cells were grown in complete DMEM

containing 10% FBS and 100 units/ml each of penicillin and streptomycin. Cells were seeded one day before transfection, at 3 x 10^5 cells per well in a 6-well plate. On the day of the transfection the cells were 70-80% confluent and were transfected with 1 µg of plasmid DNA and 6 µl of Lipofectamine™ Reagent per well. After 5 hr incubation, the transfection mixture was removed and replaced with fresh DMEM containing 10% FBS. At 48 hr after transfection, the media were changed to plain DMEM and the cells were incubated for additional 24 hr. The media were then collected and the cells were lysed in 1% SDS lysis buffer (0.1 M NaCl, 2.5 mM Tris-HCl pH 6.8, and 1% SDS). Equal volumes of media and cell lysates were boiled in SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 50 mM dithiothreitol, 0.2% bromophenol blue, and 20% glycerol), subjected to SDS-PAGE on a 4% acrylamide gel, and transferred to nitrocellulose. Immunoblotting employed rabbit polyclonal anti-Tg sera, with a goat anti-rabbit secondary antibody coupled to peroxidase. Blots were developed by using enhanced chemiluminescence with luminol. For pulse-chase experiments, metabolic labeling was started at 36 hr after transfection.

Metabolic Labeling and Immunoprecipitation

Transfected COS-7 cells were starved for 30 min in Met/Cys free DMEM, then labeled for 1 hr at 37°C with 80 µCi/ml [^{35}S]-cysteine and [^{35}S]-methionine. Following the pulse-labeling period, the cells were washed twice with PBS and chased with DMEM supplemented with an excess of cold Met/Cys, with or without 20 µM MG-132, for various durations. At the end of chase period, the media were aspirated and the labeled cells were treated with ice-cold PBS containing 50 mM iodoacetamide for 10 min to alkylate free cysteine thiols. The labeled cells were lysed in 1 ml of 1% Triton X-100 lysis buffer containing 25 mM Tris-HCl, pH 6.8, 0.1 M NaCl, 10 mM iodoacetamide, protease inhibitor cocktail (2 µg/ml aprotinin, 1.2 µM leupeptin, 11.2 µM pepstatin, 1 mM PMSF, and 5.7 mM EDTA), and 5 units/ml of apyrase when association with BiP was investigated. After a 1 hr incubation on ice, cell lysates were centrifuged at 10,000 rpm for 10 min at 4°C to remove nuclear and cellular debris. The supernatants were incubated with rabbit anti-mouse Tg antibody, anti-BiP Ab, anti-calnexin Ab, or anti-PDI Ab and zysorbin A beads overnight at 4°C. The beads were washed three times with lysis buffer and boiled in SDS sample buffer. The supernatants were then subjected to SDS-PAGE on a 4% acrylamide gel,followed by phosphorimager analysis. Tg bands were quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA, USA).

Endoglycosidase H Treatment

After the pulse-chase procedure, an aliquot of each mutant Tg transfected COS-7 cell lysate was resuspended in glycoprotein denaturing buffer (0.5% SDS and 1% β-mercaptoethanol in 20 mM Tris-HCl, pH 7.4) and boiled for 5 min. The denatured lysates were then digested with 250 units of Endo H in 50 mM sodium citrate, pH 5.5, for 1 h at 37 °C. All buffers were provided with the enzyme. The samples were then immunoprecipitated with an anti-Tg antibody followed by reducing SDS-PAGE and subsequent phosphoimager analysis.

Results

R39K Mutation in the Tg Gene is Responsible for Defective Export of Tg

To confirm that the identified R39K mutation is responsible for the defective export of Tg, an intact normal mouse Tg cDNA[27] was mutated to contain the R39K, R855Q, or V2472L mutation, all of which were identified in a patient with congenital goiter. The mouse Tg cDNA is 78% identical to human Tg cDNA and their primary amino acid sequences are 71% identical. COS-7 cells were transiently transfected with each of the mutated mouse Tg cDNAs and compared with the cells that were transfected with wild type Tg cDNA and untransfected cells. After an overnight incubation in serum-free media, the cells were lysed under denaturing conditions and analyzed by reducing SDS-PAGE. As shown by immunoblotting (Fig. 1), a specific Tg band was detected in lysates from cells transfected with the wild type and each of Tg mutants, but, as expected, not in the lysates from untransfected cells. While the wild

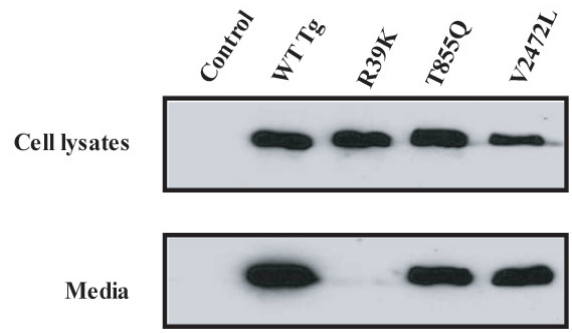

Fig 1. Transiently expressed R39K mutant Tg is retained within COS-7 cells. COS-7 cells were transiently transfected with wild type, R39K, T855Q, and V2472L mutant mouse Tg cDNAs. Forty eight hours after transfection, the cells were incubated in serum-free media for 24 h. Both the cell lysates and the secreted media were analyzed by reducing SDS-PAGE, followed by western blot with anti-Tg antibody. Control is untransfected COS-7 cells.

type Tg and R855Q and V2472L Tg mutants were seen in the media, no immuno-reactive band was observed in the medium of the R39K Tg transfected COS-7 cells. This confirms that the R39K mutation is responsible for the defective export of Tg, while the R855Q and V2472L forms could simply be polymorphisms.

The R39K Mutant Tg is Retained in the Endoplasmic Reticulum

To determine the intracellular localization of the mutant Tg, an endoglycosidase-H (Endo-H) assay was performed. Endo-H cleaves N-linked glycosyl groups with high mannose content, as long as the terminal mannose residues are not modified with other complex sugars. Since the ER contains no enzymes that add complex sugars to the N-linked glycosyl groups, all glycosyl groups on proteins localized in the ER are sensitive to Endo-H cleavage. Upon export to the Golgi, the terminal mannose residues undergo full modification with complex sugars, which causes the N-linked glycosyl group to become resistant to Endo-H digestion.

COS-7 cells transiently transfected with the R39K Tg were pulse-labeled and chased for the times indicated (Fig. 2). Upon Endo-H digestion, the R39K Tg retained intracellularly showed a mobility shift at all chase times. This indicates that the R39K Tg is retained within the ER.

Intracellularly Retained R39K Mutant Tg is Slowly Degraded by the 26S Proteasome

Since no R39K Tg was found in the secreted media, it was assumed that the mutant protein was retained intracellularly and degraded. To determine the intracellular fate of R39K mutant Tg, COS-7 cells transiently transfected with mutant Tg were pulse-labeled with ^{35}S (Met+Cys) and chased for up to 24 hours. When analyzed by reducing SDS-PAGE and quantitated by phosphorimaging, the amount of immunoprecipitated R39K Tg decreased slowly with a half time approx.10 hrs, and approx.16 % of newly made Tg still remained after the 24 hr chase (Fig. 3A and 3B). In addition, when MG-132, a proteasome inhibitor, was added to the chase media, the intracellular degradation of R39K mutant Tg was significantly

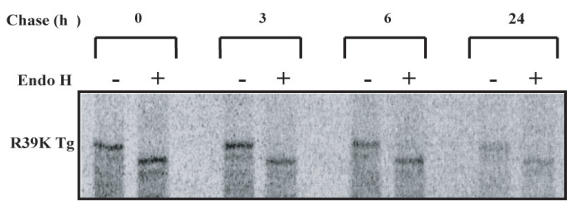

Fig 2. The R39K mutant Tg is retained in the ER. COS-7 cells transiently transfected with the R39K mutant Tg were pulse radio-labeled and chased for 3, 6, and 24 hrs. Cell lysates at different chase times were treated with Endo-H, followed by immunoprecipitation with anti-Tg antibody and subjected to SDS-PAGE on 4% acrylamide gels under reducing condition.

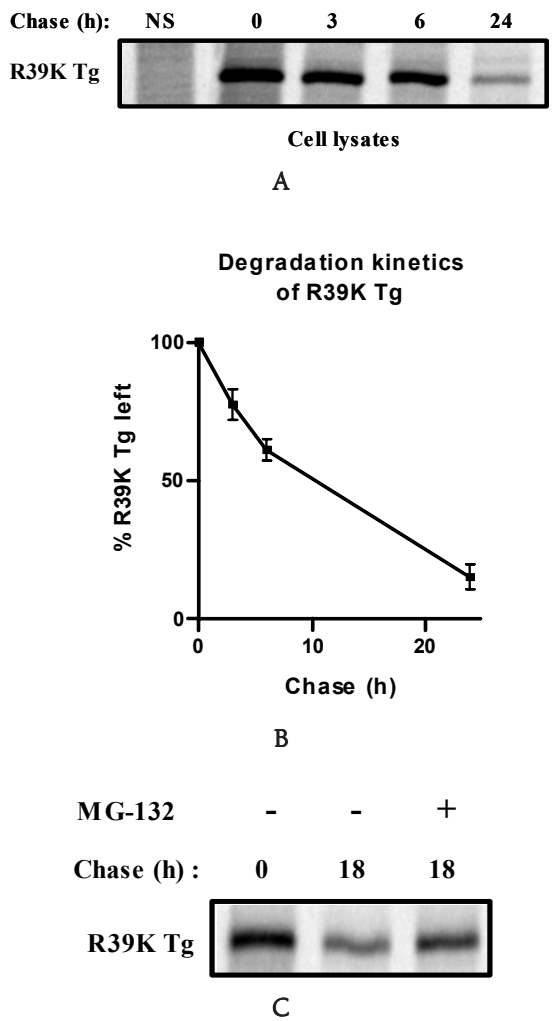

Fig 3. The R39K mutant Tg is slowly degraded by the 26S proteasome.

(A) Transiently transfected COS-7 cells expressing the R39K mutant Tg were pulse-labeled for 1 hr with ^{35}S (Met+Cys) and chased for the times indicated. Cell lysates were immunoprecipitated with anti-Tg antibody and subjected to SDS-PAGE on 4% acrylamide gel under reducing conditions. NS stands for nonspecific binding of anti-Tg antibody in the control which is untransfected COS-7 cells.

(B) Kinetics of degradation were determined by quantitation of Tg bands from three separate experiments.

(C) Transfected COS-7 cells were labeled with ^{35}S (Met/Cys) for 1 h and chased in the presence of 20 μM MG-132 for 18 hrs. Cell lysates were immunoprecipitated with anti-Tg antibody and subjected to SDS-PAGE on a 4% gel.

inhibited (Fig. 3C). This confirms that R39K mutant Tg is degraded by the 26S proteasome in the cytosol.

The Majority of Remaining R39K Mutant Tg Associates with BiP/GRP78, Calnexin, and PDI in COS-7 Cells

It has been well established that ER molecular chaperones and folding enzymes are responsible for both facilitating protein folding and retaining misfolded proteins in the early secretory pathway. BiP, a member of the HSP70 family of proteins, is thought to transiently bind to unstable hydrophobic regions of the nascent polypeptides until these regions can be buried within the core of the molecule as it folds. Therefore, BiP tends to associate strongly with misfolded proteins that are likely to have exposed hydrophobic regions. Co-immunoprecipitation of the R39K mutant Tg with a BiP-specific antibody showed significant amounts of Tg bound by this chaperone, suggesting that the R39K mutant Tg is unable to attain a correctly folded state (Fig. 4A).

We also examined the interaction between calnexin and the mutant Tg. Calnexin is thought to bind the sugar regions of the nascent glycoproteins that have not achieved their proper tertiary structures. Similar to BiP, calnexin interacted stably with the R39K mutant Tg, but at a lesser amount than BiP (Fig. 4A). This suggests an important role for the lectin pathway of ER quality control in the processing of the mutant Tg.

Both the oxidation and isomerization of disulfides on nascent polypeptides are thought to be catalyzed by protein disulfide isomerase (PDI). Since Tg contains 244 cysteines that form approximately 120 disulfide bonds, a role for PDI in the processing of R39K Tg was investigated. Co-immunoprecipitation of the R39K mutant Tg with a PDI-specific antibody showed the greatest amount of Tg is bound by this folding enzyme at all chase time (Fig. 4A), suggesting that PDI might also be a major chaperone in the intracellular processing of the mutant Tg.

Following the intracellular fate of mutant Tg by the pulse-chase experiment showed that with increasing chase time, the total amount of Tg decreased and the relative amounts of Tg bound to the three chaperones increased (Fig. 4A and 4B). Although in Fig. 4B the total percent of Tg bound to the three chaperones at 24 hr chase time appeared to be greater than 100%, this may be due to error in the densitometric measurement of the small amount of Tg remaining.

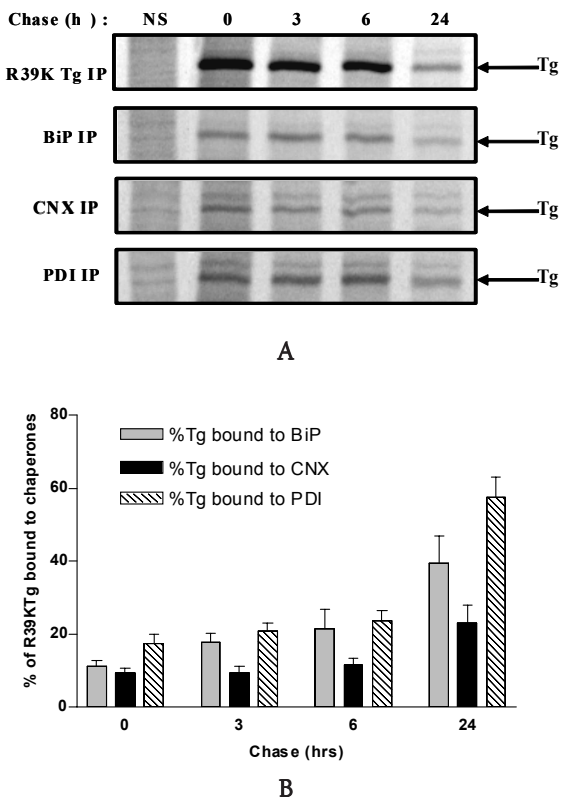

Fig 4. The R39K mutant Tg is stably bound by chaperones over long chase times.

(A) Co-immunoprecipitations of mutant Tg were carried out using anti-Tg, anti-BiP, anti-calnexin and anti-PDI specific antibodies. The immune complexes were extensively washed and dissociated by boiling in SDS sample buffer and subjected to reducing SDS-PAGE on 4% gels. NS stands for nonspecific binding of anti-Tg antibody in the control, which is untransfected COS-7 cells.

(B) Quantitation of radioactive band intensities from three separate experiments.

Discussion

Recently, three novel missense mutations in the Tg gene have been reported in a Brazilian kindred with congenital goiter and hypothyroidism[10]. These are the R39K, R855Q, and V2472L mutations. Among these, the R39K mutation is thought to cause a partial impairment of Tg synthesis, secretion or function, and while the R855Q and V2472L mutations could be simple polymorphisms. When expressed in COS-7 cells, only mouse R39K mutant Tg was not secreted but retained within the ER suggesting that the R39K mutation in the human Tg gene causes defective export of Tg and is responsible for the observed phenotype in an inbred Brazilian kindred. Although the substitution of an arginine by a lysine results in no change in amino acid charge, R39K mutation prevents normal export of Tg. Both arginine and lysine are frequently involved in salt-bridges, where they pair with a negatively charged amino acid (e.g. aspartate) to create hydrogen bonds which can be important for protein stability. Arginine

contains a complex guanidinium group in its side-chain which has a geometry and charge distribution that is able to form multiple hydrogen bonds with either a negatively charged amino acid group or non-protein group (e.g. phosphate group) while lysine contains only a single amino group that is more limited in the number of hydrogen bonds it can form. A change from an arginine to lysine in some contexts can thus be disastrous. Importantly, an arginine 39 is strictly conserved throughout all the mammalian Tgs (human, bovine, rat, and mouse). Lysine substitution for this critical arginine may disrupt a domain structure that is necessary for the correct folding of Tg.

In this study, we have also examined the intracellular fate of the R39K mutant Tg, as well as the role of several major ER molecular chaperones in processing this mutant Tg. Continued synthesis of the R39K mutant Tg, which is defective for transport, leads to the accumulation of the misfolded protein within the ER. To prevent such toxic accumulation, the mutant protein must be recognized, targeted, and transported out of the ER to the site of degradation. Using a specific inhibitor for the 26S proteasome, we have shown that the R39K mutant Tg was degraded by the proteasome, indicating that protein export from the ER to the cytosol must occur. It has been shown that both ER molecular chaperones and oxidoreductases not only facilitate protein folding, but are also responsible for stabilizing, unfolding, and, perhaps, targeting misfolded proteins for degradation[19]. A number of studies have shown a role for BiP, calnexin and PDI in the retention and degradation of misfolded proteins[28,29,30,31]. As with many other mutant secretory proteins, the R39K mutant Tg was extensively bound by BiP during the prolonged chase periods, suggesting that one or more hydrophobic segments of the misfolded R39K Tg remained exposed. Continued BiP binding is also necessary for preventing misfolded protein aggregation and keeping it in a soluble form for retrotranslocation and degradation[32].

Calnexin, an integral membrane protein of the ER, has been proposed to serve as a major ER retention factor for certain misfolded glycoproteins[29]. Although calnexin has been previously shown to play a lesser role in posttranslational ER retention/quality control for Tg[33], we have found that the mouse R39K mutant Tg has a prolonged association with calnexin. This suggests an important role for the lectin pathway of ER quality control in the processing of the mutant Tg. Quantitatively, calnexin was co-immunoprecipitated with the mutant Tg in a lesser amount than BiP. A possible explanation may be that the synthesis of calnexin is not affected by the unfolded protein response[34]. Therefore, the competition for the binding sites on misfolded protein would not favor enhanced calnexin binding when the concentrations of lumen chaperones increased.

In addition to BiP and calnexin, we have examined the possible chaperone role of PDI, which normally functions as a redox foldase. PDI has also been reported to mediate retrotranslocation of a cysteine-free misfolded protein for proteasome degradation, suggesting a chaperone-like role for PDI in ER-associated degradation[35]. In this study, PDI showed the greatest prolonged stable association with the mouse R39K mutant Tg. These results provide new evidence that PDI may also be a chaperone in the intracellular processing of the mutant Tg.

It has been shown that degradation of misfolded proteins is inhibited if the interaction with chaperones is disrupted[35,36,37]. Therefore, prolonged chaperone binding to the R39K mutant Tg after unsuccessful folding trials may be essential for targeting mutant Tg for ER-associated degradation. However, the release of chaperones from misfolded proteins is required in order for retro-translocation and degradation to take place. Therefore, association between chaperones and a fraction of mutant Tg at late chase time (24 hrs) could have attenuated the degradation process of that fraction of mutant Tg.

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