

An Endoplasmic Reticulum Storage Disease Causing Congenital Goiter with Hypothyroidism

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Abstract. In humans, deficient thyroglobulin (Tg, the thyroid prohormone) is an important cause of congenital hypothyroid goiter; further, homozygous mice expressing two *cog/cog* alleles (linked to the Tg locus) exhibit the same phenotype. Tg mutations might affect multiple different steps in thyroid hormone synthesis; however, the microscopic and biochemical phenotype tends to involve enlargement of the thyroid ER and accumulation of protein bands of $M_r < 100$. To explore further the cell biology of this autosomal recessive illness, we have examined the folding and intracellular transport of newly synthesized Tg in *cog/cog* thyroid tissue. We find that mutant mice synthesize a full-length Tg, which appears to undergo normal N-linked glycosylation and glucose trimming. Nevertheless, in

the mutant, Tg is deficient in the folding that leads to homodimerization, and there is a deficiency in the quantity of intracellular Tg transported to the distal portion of the secretory pathway. Indeed, we find that the underlying disorder in *cog/cog* mice is a thyroid ER storage disease, in which a temperature-sensitive Tg folding defect, in conjunction with normal ER quality control mechanisms, leads to defective Tg export. In relation to quality control, we find that the physiological response in this illness includes the specific induction of five molecular chaperones in the thyroid ER. Based on the pattern of chaperone binding, different potential roles for individual chaperones are suggested in glycoprotein folding, retention, and degradation in this ER storage disease.

DEFICIENT thyroglobulin (Tg)¹ is estimated to cause congenital goiter and hypothyroidism with a prevalence of $\sim 1/40,000$ humans, and similar pathology has been described in numerous animal models (Medeiros-Neto et al., 1993; Medeiros-Neto and Stanbury, 1994). Affected individuals may exhibit a range of phenotypes, including hypothyroid growth retardation, abnormal central nervous system function, and local compression of neck tissues due to an enlarged thyroid mass. In mice, a defective *cog* gene accounts for the development of the same autosomal recessive phenotypes (Beamer et al., 1987; Sugisaki et al., 1992); the *cog* gene is linked to the Tg locus (Taylor and Rowe, 1987) and appears to represent a mutation in the Tg gene product (Adkison et al., 1990).

In humans as well as animal models of this illness, postulated Tg-related defects have included abnormal mRNA

splicing, translation, protein transport, posttranslational modifications, or exocytosis. Interestingly, a frequently described microscopic and biochemical phenotype involves distention of the thyroid ER (Michel-Bechet et al., 1969; Lissitzky et al., 1975; Mayerhofer et al., 1988) and accumulation of protein bands ($M_r < 100$) that are too small to represent full-length Tg (Basche et al., 1989; Adkison et al., 1990; Fogelfeld et al., 1992; Ohyama et al., 1994).

Because protein iodination leading to thyroid hormone synthesis has been observed on Tg homodimers at the end-stage of the secretory pathway (Dunn, 1992; Kuliawat and Arvan, 1994), we have been interested in understanding the process of Tg folding that leads to homodimerization. Using cultured porcine thyrocytes, we recently have established that nascent Tg monomers are highly unfolded and susceptible to protein aggregation, before these monomers achieve a sufficiently folded conformation to allow stable homodimers to form (Kim et al., 1992). It was once considered possible that dimerization might occur as a late, perhaps postsecretory event (Lissitzky et al., 1964; Seed and Goldberg, 1965; Cavalieri and Searle, 1967; Vecchio et al., 1972; Kondo and Kamiya, 1976). However, it has been suggested that because of machinery regulating the quality control of protein export, homotypic assembly of newly synthesized subunits in the ER may be required for normal Tg secretion (Kim and Arvan, 1991).

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1. *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; endo H, endoglycosidase H; ERSD, ER storage disease; Tg, thyroglobulin.

With these ideas in mind, we have examined closely the folding and assembly of newly synthesized Tg in *cog/cog* mice. We find that in the mutant, newly synthesized Tg is a full-length translation product which appears to undergo normal N-linked glycosylation, yet, based on a temperature-sensitive conformational defect, a deficient quantity of Tg homodimerizes and exits the ER of thyrocytes. The hypothyroidism and congenital goiter in *cog/cog* mice therefore represents one of a growing family of ER storage diseases. As a consequence of this perturbation of normal thyroid cell biology, we have been able to examine the regulation and roles of specific ER chaperones in the physiological setting.

Materials and Methods

Animals

A breeding pair of *cog/cog* mice and wild-type littermates were initially obtained from the Jackson Laboratories (Bar Harbor, ME). At 2 mo of age, the mutant mice were hypothyroid (serum thyroxine <1.0 µg/dl) compared with the genetically identical background strain (~4.0 µg/dl). We observed no difference in Tg folding and export between this wild-type genetic background and the C-1 strain (not shown); in most experiments, thyroids from the C-1 strain were used routinely to represent normal mice. To eliminate pituitary stimulation of the thyroid, where indicated, both sets of animals were treated (12–16 wks) with 1 µg/ml thyroxine in the drinking water.

Preparation of Thyroid Tissue

Excised mouse thyroid glands were obtained from euthanized mice and analyzed fresh. The thyroids were trimmed of connective tissue and chopped into 1-mm cubes, each containing only a few follicles (Kim et al., 1993), or further minced so that some of the follicles were broken (Fig. 2B). The minced tissue was washed at least three times in ice-cold Dulbecco's PBS to remove debris and colloidal contents from follicles injured during tissue preparation.

Antibodies

Based on the knowledge that the extreme NH₂ terminus of mature Tg is well conserved between different species (Rawitch et al., 1984; Dunn et al., 1987), a peptide was synthesized representing the first 15 amino acids of rat Tg, and this peptide was coupled to the activated carrier protein acceptor, KLH (Pierce, Rockford, IL). A polyclonal antibody directed against this peptide was raised in chicken, and IgY purified from eggs was generated for use in subsequent studies. HRP-conjugated anti-chicken-IgY (Sigma Chemical Co., St. Louis, MO) was used for immunoblotting with enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL).

Polyclonal anti-chaperone antibodies were generated as follows: COOH-terminal synthetic peptides beginning with an introduced cysteine (14 residues of murine GRP94, 16 residues of murine BiP, 19 residues of rat ERp72 excluding the final 3 residues, 18 residues of rat ER60, 15 residues of murine calreticulin, and 19 residues of murine calnexin) were protein conjugated and used for immunizing rabbits. The last 18 residues of rat ER60 were prepared similarly for immunization in mice. Each of these antisera (except that against calnexin, which lacks a KDEL-tail) was first passed through a column covalently bound to the peptide CAVKDEL (thereby removing cross-reacting antibodies); the unbound portion of these sera were then affinity purified using a similar resin coupled to the corresponding peptides listed above. Unpurified sera were suitable for Western blotting; affinity purified sera were used for coprecipitation studies. Rabbit antisera directed against murine protein disulfide isomerase (PDI) (affinity purified against the last 17 residues) and rat ribophorin I were obtained from Drs. T. Wileman (Pirbright Laboratories, Surrey, UK) and G. Kreibich (New York University, NY), respectively.

Hormones and Other Reagents

Bovine and ovine thyrotropin (TSH) were obtained from Dr. A.F. Parlow via the National Hormone and Pituitary Program. Recombinant endogly-

cosidase H (endo H) was from New England Biolabs Inc. (Beverly, MA). Protease inhibitors, iodoacetamide, tunicamycin, brefeldin A, and dithiothreitol were from Sigma Chemical Co., castanospermine was from Boehringer Mannheim Biochemicals (Indianapolis, IN), Zysorb-Protein A was from Zymed Labs, Inc. (San Francisco, CA), and Omnisorb-Protein G was from Calbiochem Corp. (La Jolla, CA).

Cell Labeling and Lysis

For each tissue preparation, samples were incubated for 30 min at 37°C in DME lacking methionine and cysteine. The tissue was then labeled for up to 30 min with [³⁵S]amino acids (Expres³⁵S, New England Nuclear, Boston, MA) for the times stated, and chased in the presence of excess unlabeled methionine and cysteine. Where indicated, prolonged incubation was performed to collect chase media which contained secreted Tg. Tunicamycin, when used, was used at a final concentration of 20 µg/ml for 3 h before labeling (Kim et al., 1992). Castanospermine was used at the maximum soluble dose (1.32 mM) for 4 h before labeling. Brefeldin A was used at 10 µg/ml. At the conclusion of each chase period, the cells were treated with 50 mM iodoacetamide in ice-cold PBS for 10 min to alkylate sulfhydryls intracellularly (Braakman et al., 1991; Lodish et al., 1992; Kim et al., 1993). In some experiments, the cells were then lysed directly in an SDS-containing buffer. In preparation for two-dimensional PAGE, the cells were washed three times in ice-cold PBS and then lysed by brief sonication using a sonifier with cup-horn (Branson Ultrasonics Corp., Danbury, CT) in 0.5 ml of a solution containing 0.1 M NaCl, 25 mM Tris, pH 6.8, 5 mM EDTA, 0.1% Triton X-100, a cocktail of protease inhibitors (0.1 mM leupeptin, 10 mM pepstatin, 1 mM diisopropylfluorophosphate, 1 mU/ml aprotinin), and 20 U/ml apyrase for 60 min before addition of 10 mM iodoacetamide. For experiments in which Tg was to be coimmunoprecipitated with GRP94, thyroid tissue before lysis was exposed to alkylating agent plus 0.2 mM disuccinimidyl propionate (Melnick et al., 1992). The cells were incubated in the reducible cross-linker (Pierce) for 30 min at 4°C, just before and for 30 min after lysis at 4°C; in this case the lysis buffer omitted apyrase.

Immunoprecipitation

Equal aliquots of cell lysates were first precleared with Zysorb or Omnisorb and then immunoprecipitated with each anti-chaperone antibody for 2 h at 4°C, before analysis by reducing SDS-PAGE.

Endoglycosidase H Digestion

Endo H digestion was performed essentially as previously described (Arvan and Lee, 1991), using Tg that had been reduced and alkylated after SDS denaturation.

Gel Electrophoresis and Quantitation

Reducing, nonreducing SDS-PAGE, and two-dimensional PAGE were essentially as previously described (Kim and Arvan, 1991). In most instances, before gel loading, samples were normalized either to DNA content or to comparable amounts of labeled Tg. Where indicated, the gels were dried by conventional methods and visualization and quantitation of radiolabeled Tg bands were performed by phosphorimaging coupled to the ImageQuant software package (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Identification of Newly Synthesized Mouse Tg

Tg is the most abundant thyroid protein, representing in certain conditions up to 75% of total glandular protein (Van Herle et al., 1979); further, the fraction of new protein synthesis devoted to Tg is higher than for any other thyroid polypeptide (Wagar, 1974; Dumont et al., 1989). This, coupled with the uniquely high molecular weight of ~330,000 per monomer polypeptide, makes Tg readily identifiable after amino acid radiolabeling, even without immunoprecipitation of cell lysates (see Fig. 8 A, Kim and Arvan, 1991) or secretion (see Fig. 1, Kim and Arvan,

1993). To confirm this point, fresh thyroid tissue from normal mice was briefly pulse labeled with [³⁵S]amino acids. The labeled cellular homogenate was analyzed by SDS-PAGE and phosphorImaging (Fig. 1, lane 1). Immunoprecipitation from the homogenate with anti-Tg selectively recovered a radiolabeled band of 330,000. Although a single round of immunoprecipitation recovered only 60–90% of this band (see Fig. 1, lane 2), a repeat round of precipitation with anti-Tg recovered the remaining fraction (lane 3). At this time, the supernate representing the cleared homogenate was devoid of labeled ~330-kD band, indicating that all of the radiolabeled material originally found in the homogenate at this position (lane 1) was comprised of Tg. The unmistakable identification, even without immunoprecipitation, of pulse labeled Tg from mouse thyroid tissue, greatly accelerated the analysis of subsequent experiments.

Quantitative Defect in Tg Production in Thyroid Glands from Mutant Mice

Since *cog/cog* mice in this study are homozygotes, the polypeptide synthesized from either Tg allele is expected to yield an identical translation product. With this in mind, we compared the electrophoretic behavior of newly synthesized Tg from fresh thyroid tissue of normal and *cog/cog* mice after a brief pulse labeling. The *cog/cog* thyrocytes synthesized a Tg band that precisely comigrated with the intact, full-length Tg glycoprotein of normal mice (Fig. 2 A, lanes 1 and 3), although when corrected for cell number, there was diminution in the intensity of this band. Further, when N-linked glycosylation was inhibited by treatment of thyroid tissue with tunicamycin, the Tg polypeptide moiety from *cog/cog* mice also comigrated with the unglycosylated version of normal Tg (Fig. 2 A, lanes 2 and 4), indicating that Tg receives similar N-linked core glycosylation

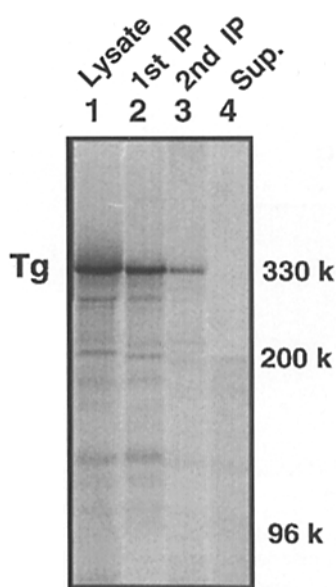


Figure 1. Identification of newly synthesized mouse Tg in thyroid tissue from normal mice. Normal mouse thyroid tissue was pulse-labeled for 30 min with [³⁵S]amino acids and lysed without chase. One-half of the lysate was diluted into SDS-gel sample buffer (*lysate*, lane 1) and a second half was exposed to anti-Tg for a first (*1st IP*, lane 2) and then a second (*2nd IP*, lane 3) round of immunoprecipitation. One-tenth of each of these samples was analyzed by 4–10% gradient SDS-PAGE, along with one-tenth of the remaining supernate (*Sup.*, lane 4) at the conclusion of the sequential immunoprecipitation. The bands were visualized by

phosphorImaging. The positions of molecular weight standards are shown at right. 330 k, secreted Tg; 200 k, myosin; 96 k, phosphorylase B.

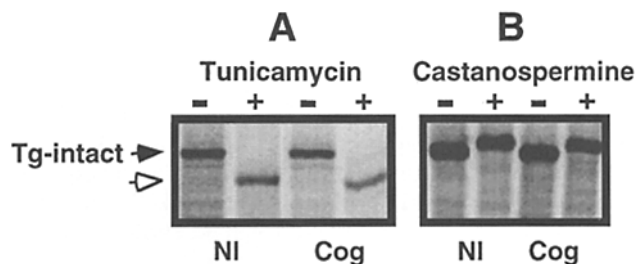


Figure 2. Synthesis and glycosylation of Tg in thyrocytes of normal and *cog/cog* mice. Normal and mutant mouse thyroid tissues were pulse-labeled with [³⁵S]amino acids and analyzed by 4% SDS-PAGE and phosphorImaging after loading for comparable amounts of labeled Tg. Both tissues are seen to contain full-length Tg. (A) Regardless whether pretreated or not pretreated with tunicamycin, no difference could be detected for the mobilities of glycosylated (*closed arrow*) or unglycosylated (*open arrow*) Tg between normal (*NI*) and mutant (*Cog*) thyrocytes, suggesting that both sets of Tg receive the same N-linked glycans. (B) Thyroid tissues, pretreated or not pretreated with castanospermine, were labeled as in (A), and analyzed by SDS-PAGE after immunoprecipitation with anti-Tg. No difference could be detected for the mobilities of glucose trimmed or untrimmed Tg between normal (*NI*) and mutant (*Cog*) thyrocytes, indicating that both sets of Tg receive the same degree of glucose trimming of their N-linked glycans.

in both cases. Moreover, when glucose trimming of Tg by glucosidases I and II was inhibited with castanospermine (Franc et al., 1990), the shift-up in mobility of newly synthesized Tg was the same for both normal and mutant (Fig. 2 B). Thus, not only is Tg from *cog/cog* mice synthesized as a full-length intact protein, it also undergoes N-glycosylation and glucose trimming that is indistinguishable from normal. Nevertheless, upon subsequent chase for up to 16 h at 37°C, secretion of labeled Tg from mutant thyroid tissue was below the limits of detection in our assay (Fig. 3).

Export of Tg from the ER to the Distal Secretory Pathway Is Inhibited in *cog/cog* Mice

Since labeled Tg from the mutant exhibited defective secretion, we attempted to determine the intracellular loca-

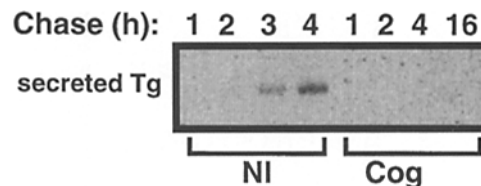


Figure 3. Secretion of Tg from thyrocytes of normal and *cog/cog* mice. Thyroid tissue was finely minced and pulse-labeled with [³⁵S]amino acids as described in Materials and Methods. Upon further chase, the labeled Tg released into the medium from broken follicles (*secreted Tg*) was analyzed by 4% reducing SDS-PAGE and phosphorImaging. Unlike normal thyroid tissue, there is no detectable secretion of newly synthesized Tg from the mutant at 4 h or even 16 h chase at 37°C; although this is not an ultrasensitive assay, we did not detect Tg released into the medium even at our longest exposure.

tion of this defect. It is well known that upon export to the Golgi complex, a subset of the glycans on each Tg molecule undergoes full Golgi-carbohydrate modification that cause these particular glycans to become resistant to digestion with endo H (Arvan and Lee, 1991). Indeed, in normal mouse thyroid tissue after a long pulse labeling at 37°C, loss of endo H sensitivity was already underway (Fig. 4 A). In addition, recent studies have shown that conformationally mature Tg has a high susceptibility to limited endoproteolysis in a short stretch of residues in the mature protein sequence that is concentrated immediately after position 500 and is hypothesized to represent a physiologically relevant cleavage region. Endoproteolysis in this region generates predicted small NH₂- and large COOH-terminal fragments (Gentile et al., 1992; Gentile and Salvatore, 1993) that we have termed Tg-frag₁ and Tg-frag₂, respectively. Interestingly, as a consequence of further chase of normal mouse thyroid tissue at 37°C, not only did the endo H-sensitive form of Tg decrease, but a large radiolabeled Tg fragment was produced that corresponded in size to Tg-frag₂ (Fig. 4 B); all three forms shown were immunoprecipitable with a polyclonal anti-Tg (not shown). Upon endoglycosidase digestion, Tg-frag₂ underwent a mobility shift paralleling that observed for endo H-resistant Tg (Fig. 4 B); these data indicate that the endoproteolytic cleavage to generate Tg-frag₂ occurs after Tg export to the Golgi. Consistent with this, we found that if, after a long pulse labeling in normal mouse thyroid tissue, the tissue was then chased in the presence of brefeldin A to block further ER export, the endo H-sensitive population of Tg molecules found at the conclusion of the pulse (Fig. 4 A) did not shift all the way up to the position of the Golgi form during the chase (Fig. 4 C), nor was labeled Tg-frag₂ detectable (Fig. 4 C).

Importantly, in the mutant at 37°C (in the absence of brefeldin A), Tg remained essentially completely endo H sensitive at chase times up to 4 h, and there was no detectable endoproteolytic production to yield Tg-frag₂ (Fig. 4 D).

We also manufactured an antibody directed against the highly conserved Tg NH₂ terminus, as a means to increase the sensitivity for detection of Tg-frag₁.² By Western blotting (Fig. 5), the band showing the most intense NH₂-terminal immunoreactivity, corresponding to Tg-frag₁ (M_r ~60–65), was detected only in normal but not in mutant mice. (As an interesting aside, while a number of higher molecular weight immunoreactive Tg fragments overlapped between the two specimens, the mutant thyroid reproducibly exhibited a smaller NH₂-terminal Tg fragment of M_r ~45,000 that was not detected in normal thyroid tissue [asterisk, Fig. 5]). Thus, taken together, the data strongly suggest that in *cog/cog* mice, the amount of Tg exiting the thyrocyte ER is profoundly inhibited, leading to a markedly decreased fraction of Tg that acquires endo H resistance or undergoes endoproteolytic cleavage to Tg-frag₂ (Fig. 4) and Tg-frag₁ (Fig. 5).

Tg in cog/cog Mice Exhibits a Temperature-sensitive Defect in Folding/Dimerization

It is now established that the major feature limiting protein export from the ER is “quality control”, in which unfolded and unassembled exportable proteins, recognized by structural criteria, are retained and degraded in proximal compartments of the secretory pathway (Hammond and Helenius, 1995). To see if quality control might be a part of the *cog* phenotype, we first examined conformational maturation of newly synthesized mouse Tg, using a two-dimensional PAGE system. With this system it has already been established for cultured porcine thyrocytes that in the first (native) dimension, unfolded monomers migrate as a broad smear; folded monomers accumulate

2. Detection of stoichiometric quantities of Tg-frag₁ by radiolabeling or protein staining is more difficult because it contains approximately fourfold less incorporated label than Tg-frag₂, and is poorly stained with Coomassie blue.

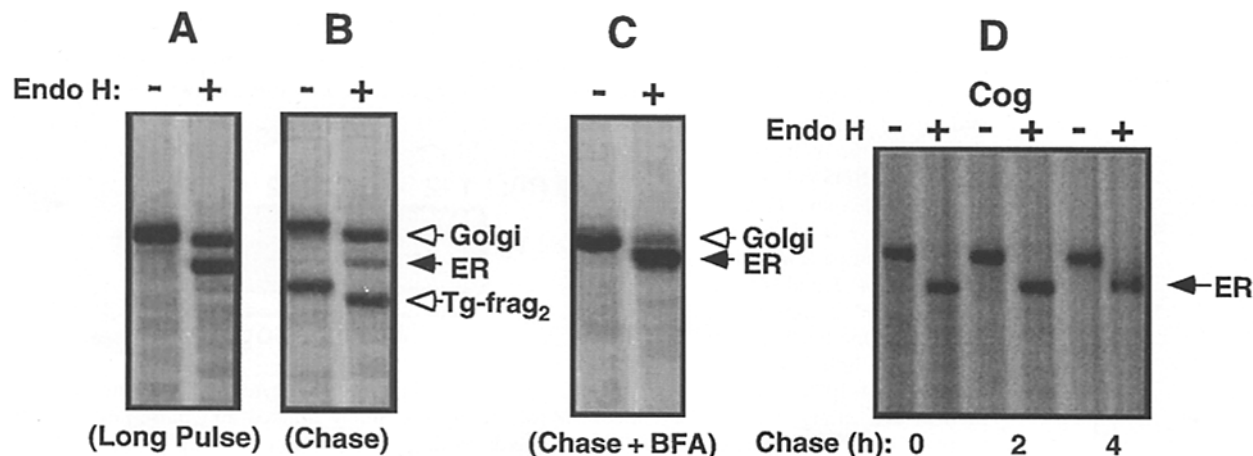


Figure 4. Retention of Tg in the ER of *cog/cog* thyrocytes. Minced thyroid tissue from normal mice, pulse-labeled as in Materials and Methods (A), or chased at 37°C for 6 h in the absence (B) or presence of BFA (C), were digested with Endo H, and analyzed by SDS-PAGE and phosphorImaging. Endo H-sensitive (ER) and resistant (Golgi) forms of intact Tg, as well as Tg-frag₂ (which appears Endo H resistant, see text) are shown. In D, minced thyroid tissue from the mutant was pulse-labeled and chased at 37°C without drugs for the times indicated. The fraction of newly synthesized Tg that acquired Endo H resistance or endoproteolysis to Tg-frag₂ is virtually undetectable at 37°C.

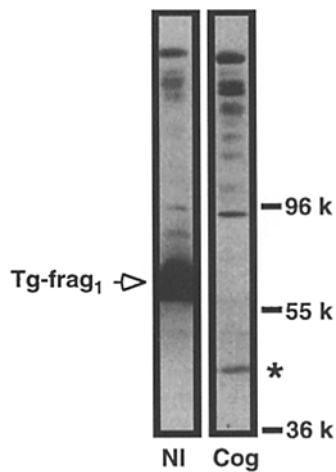


Figure 5. Immunoblotting of normal and mutant thyroid homogenates with an antibody designed to detect endoproteolytic fragments containing the Tg NH₂ terminus. Thyroid homogenates were analyzed by 8% reducing SDS-PAGE before transfer to nitrocellulose for immunoblotting with the Tg NH₂-terminal antibody described in the text. A major band of M_r ~60–65, corresponding to Tg-frag₁ (see text), was detected only in normal thyroid tissue (NI). By contrast, a Tg-fragment of M_r ~45 kD

(*) was notably increased only in the mutant (Cog). Full-length Tg is not seen in this figure because it fails to transfer from the top of the 8% resolving gel under the electrophoretic conditions used to optimally transfer the fragments. However, after prolonged transfers of these samples from 4% gels, full-length Tg was readily detected (not shown). The positions of molecular weight standards are shown at right. 96 k, phosphorylase B; 55 k, glutamate dehydrogenase; 35 k, lactate dehydrogenase.

very little but rapidly assemble to dimers which migrate as a discrete spot; and dimers represent the form leaving the ER and migrating to the Golgi complex for terminal glycosylation (Kim and Arvan, 1991; Kim et al., 1992). Of course, in the second (SDS-PAGE) dimension, essentially all Tg runs with similar mobility, on a horizontal line based on a monomer molecular weight of ~330,000.

As shown in the left set of panels of Fig. 6 after 30 min of continuous radiolabeling, newly synthesized normal mouse Tg was distributed over a wide region in the first dimension, indicating a myriad of unfolded conformational states, although there was already detectable formation of labeled dimers (closed arrow). After further (0.5 h) chase, the unfolded forms of Tg were greatly attenuated, and augmentation of Tg dimers in the ER was evident. By 1 h, a significant portion of Tg dimers had already migrated to the Golgi complex, where they underwent a mobility shift as a consequence of further carbohydrate processing (open arrow). In the mutant at zero chase, the appearance was similar to normal (although formation of Tg dimers was below the limit of detection, Fig. 6, right upper panel). However, there was dramatic persistence of unfolded Tg, such that even at 1 h, labeled Tg dimers were not appreciated (Fig. 6, right middle panel). Indeed by 2 h, a change in distribution appeared to be underway such that labeled Tg was accumulating abnormally near the top of the native gel (arrowhead, right lower panel). These data suggest that a large fraction of newly synthesized Tg in the mutant thyroid fails to attain a normal tertiary and quaternary structure.

Despite the apparent severity of the defect, published evidence has confirmed that a small fraction of newly synthesized Tg is in fact exported from the ER in *cog/cog* mice, for two reasons. First, sensitive radiolabeling experiments with ¹²⁵I⁻ in homozygous mutant mice have suggested that a small amount of Tg undergoes iodination

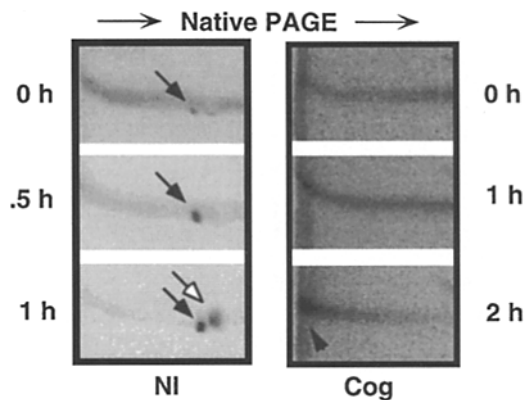


Figure 6. Tg in *cog/cog* mice exhibits a defect in tertiary and quaternary structural maturation. Cone-down views of two-dimensional (native/SDS) PAGE show that after a 30-min pulse, normal Tg (NI) is essentially fully dimeric by 1 h of chase at 37°C (closed arrows) and begins to migrate to the Golgi (open arrow). The identities of ER and Golgi forms of Tg dimer were confirmed by digestion with endo H. In the mutant (Cog), dimeric forms are absent, and by 2 h, the distribution of Tg shifts towards the top of the native dimension (arrowhead). Radiolabeled Tg bands were visualized by phosphorimaging; exposures of the gels in the mutant were increased to enhance sensitivity for the possible detection of Tg dimers.

(Fogelfeld et al., 1992); this covalent modification occurs only after Tg export from the ER (Kuliawat and Arvan, 1994). Second, analogous to human congenital euthyroid goiter (Ohyama et al., 1994), even without treatment with exogenous thyroid hormone, *cog/cog* mice ultimately attain normal blood concentrations of thyroid hormone (Adkison et al., 1990) derived from Tg (Basche et al., 1989). Therefore, we can surmise that the Tg defect is not maximally severe. Since partial misfolding (e.g., as is found in point mutations or glycosylation defects) is occasionally temperature-sensitive (Gibson et al., 1979; Manchester et al., 1994), we reexamined Tg export from the thyrocyte ER at the reduced temperature of 31°C, using a pulse-chase protocol. In this case at 2 h of chase, a readily detectable (albeit small) fraction of newly synthesized Tg now acquired endo H resistance (Fig. 7). Moreover, by 4 h of chase at 31°C, the endo H-resistant full-length Tg became endoproteolytically converted to Tg-frag₂. Although this occurred for only a minority of the labeled Tg population, the evidence indicates that *cog/cog* thyrocytes express a thermosensitive mutant Tg, which diminishes the quantity of protein that progresses to a conformation allowing escape from quality control regulation, leading to an ER storage disease (ERSD) (Callea et al., 1992).

Role of Specific ER Chaperones in Quality Control in the Thyroid ERSD

When comparing the overall Coomassie blue-stained profile of protein extracts from mouse thyroid tissue, in addition to intact Tg (present in both normal and mutant thyroid) as well as Tg-frag₂ (present only in normals), a group of bands ranging from 50–100 kD are dramatically enriched in the mutant thyroid (Adkison et al., 1990; and our unpublished observations). Because ER-specific molecu-

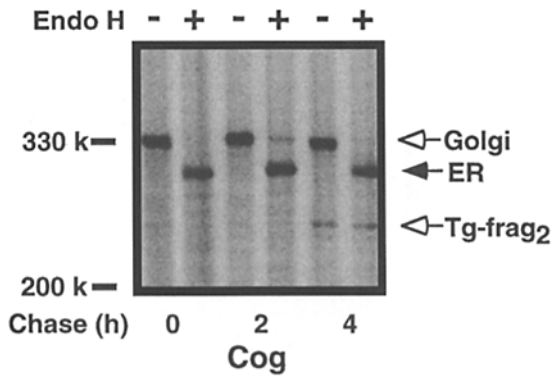


Figure 7. The defect in *cog/cog* mice is temperature sensitive. In the mutant chased at 31°C for 2 h, a detectable fraction of Tg becomes resistant to digestion with Endo H (Golgi form). In addition, the appearance of Tg-frag₂ is subsequently detectable. Both features indicate Tg export from the ER. When compared with Fig. 4 B, it is evident that for the small fraction of mutant Tg that escapes the ER, more of the N-glycans undergo Golgi-type carbohydrate processing (i.e., less Endo H mobility shift) than for normal Tg. This suggests persistent structural differences between the two types of Tg even after ER export. The position of molecular weight standards are shown at left.

lar chaperones are thought to be quality control factors that influence the efficiency of protein export and protein degradation, and because the observed Coomassie bands have calculated molecular masses (~94, ~78, ~72, ~61, and ~58 kD, [Adkison et al., 1990; and our unpublished observations]) that could be consistent with known ER resident proteins (GRP94, BiP, ERp72, ER60, and calreticulin, respectively), we wanted to examine more closely these chaperones that might play a part in physiologic compensation for this thyroid ERSD. For this purpose, both mutant and control mice were treated with full replacement doses of exogenous thyroid hormone in the drinking water for at least 12 wks to minimize any contribution of hormonal stimulation of the thyroid gland that can alter ER chaperone levels (Kim and Arvan, 1993). Antibodies (either prepared or obtained against an assortment of ER resident proteins) were then used for immunoblotting of normal and mutant thyroid tissue samples.

When normalized to cellular DNA, immunoblotting with ECL (Fig. 8 A) could be used to quantitate the levels of ER proteins in thyroid homogenates (Fig. 8 B). Because ER expansion is a known feature of this disease (Mayerhofer et al., 1988) and because the unfolded protein response may be coupled to ER biogenesis (Cox et al., 1993), it was not surprising that ribophorin I (a subunit of oligosaccharyl transferase, Kelleher et al., 1992)—known to be a reliable marker of ER size (Rajasekaran et al., 1993)—rose 2–2 1/2-fold in *cog/cog* thyrocytes, while protein disulfide isomerase (a luminal ER foldase, Freedman et al., 1994) and calnexin (a membrane-bound chaperone, Ou et al., 1993) were increased in the same range (Fig. 8 B). However, in the thyroids of mutant mice, the concentrations of a selective subset of five ER residents increased substantially above this level (Fig. 8 B). Indeed, we observed increases in the levels of ER chaperones endoplasmic/GRP94 (nearly fivefold), BiP/GRP78 (approximately

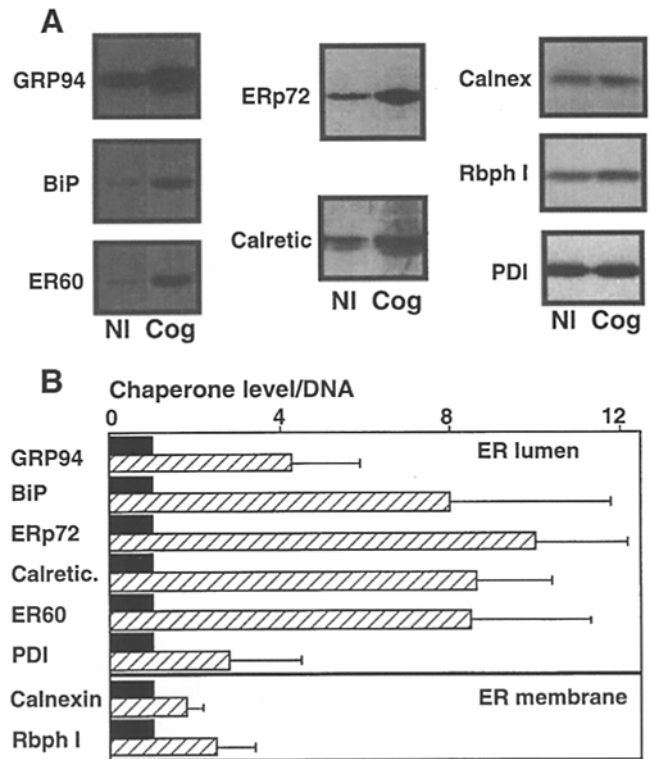


Figure 8. Accumulation of ER resident proteins in thyrocytes of *cog/cog* mice includes induction of specific molecular chaperones. Thyroid homogenates, normalized to DNA, were examined by immunoblotting, followed by quantitation of specific protein levels. (A) Antichaperone sera, described in Materials and Methods, were used for immunoblotting with enhanced chemiluminescence. (B) Using a minimum of three separate exposures for each immunoblot to ensure that linearity of the x-ray film was not exceeded, appropriate films were selected for quantitation of band intensities by scanning densitometry. Quantitation from a minimum of three different experiments indicates that five ER resident proteins were induced 5–10-fold when compared with controls (error bars, SD). Less dramatic increases were observed in PDI, calnexin, and ribophorin I (Rbph I), consistent with a generalized increase in ER mass. Arb. units: ■, NI, ▨, cog.

eightfold), and calreticulin (approximately ninefold). Moreover, ER60 and ERp72, two proteins implicated in ER protein degradation (Urade et al., 1993; Otsu et al., 1995) were similarly increased (approximately ninefold and approximately tenfold, respectively). Because no increase in any of these proteins was seen when comparing other tissues of mutant and normal mice (data not shown), and the elevation of other thyroid ER proteins did not exceed that observed for ribophorin I, the data in Fig. 8 B implicate these five ER residents in particular as part of a specific compensatory response to this thyroid ERSD.

To examine the roles of the ER members of the hsp90 and hsp70 families, we followed the interaction of newly synthesized Tg with these chaperones by coimmunoprecipitation after pulse radiolabeling. As previously established for GRP94, chemical cross-linking was necessary to efficiently preserve its interactions with exportable proteins (Melnick et al., 1992; 1994), although cross-linking was not used for other ER resident proteins (Fig. 9 A). In

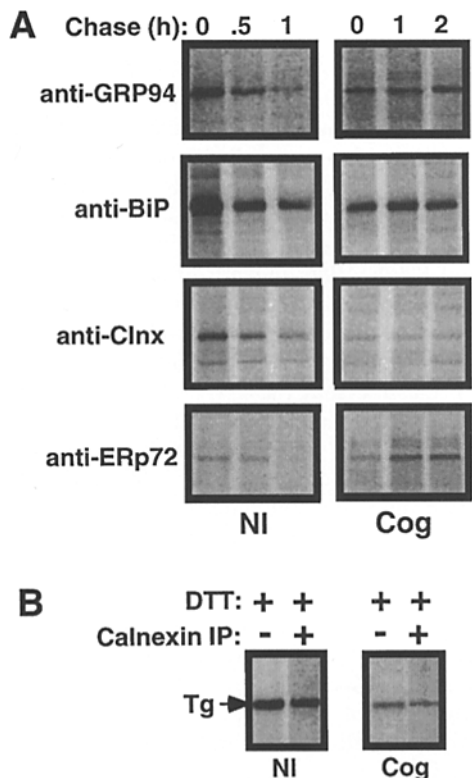


Figure 9. Structurally deficient Tg from *cog/cog* mice exhibits long-term association with GRP94 and BiP but not calnexin, while ERp72 binding shows a novel pattern. (A) For analysis of Tg coprecipitation with the chaperones, thyroid homogenates, normalized to DNA, were immunoprecipitated with affinity-purified anti-chaperone antibodies. Recovery of newly synthesized Tg was analyzed by reducing SDS-PAGE and quantitated by phosphorimaging. In the one case of anti-GRP94, thyrocyte proteins were cross-linked before lysis as described in the Materials and Methods. Direct recoveries of GRP94, BiP, and calnexin were all $\geq 75\%$ by immunoprecipitation, whereas for ERp72, recovery was $\leq 50\%$. (B) Pulse-labeled thyrocytes were briefly exposed to 25 mM DTT (10 min) before wash out of the reducing agent and lysis as described (Kim and Arvan, 1995). Labeled Tg recovered from the whole cell lysate (-) was compared with that coprecipitated with anticalnexin (+). Quantitative Tg coprecipitation with anticalnexin in normal and mutant thyroid tissue indicates that in both cases, calnexin and Tg are potentially compatible binding partners.

the normal thyroid, the kinetics of dissociation of GRP94 and BiP was largely overlapping, declining substantially over 60 min of chase. Unlike this, in the mutant, Tg association did not diminish over 2 h. These data correlated closely with the kinetics of successful folding in the normal, and incomplete folding/homodimerization in the mutant (Fig. 6). Such observations are consistent with potential roles for GRP94 and BiP, expressed within the physiologic context, in the inhibition (i.e., quality control) of Tg export from the ER (Kim et al., 1992).

We were also interested to examine calnexin, because previous kinetic studies have suggested that most of the important interactions of this chaperone with Tg are completed during polypeptide translocation into the ER lumen (Kim and Arvan, 1995); thus, one might not expect

this chaperone to serve a quantitatively significant role in quality control of Tg export during the posttranslational period. By contrast, this molecule has been proposed to serve as a major ER retention factor for certain misfolded glycoproteins (Helenius, 1994), because they may be repeatedly reglycosylated (Sousa et al., 1992) to stimulate chaperone association (Hebert et al., 1995). Tg, with 14–22 N-linked oligosaccharides (Arima et al., 1972), is a well-known substrate for the ER deglycosylation/reglycosylation cycle (Parodi et al., 1983); and based on studies of model multiglycosylated proteins, a large fraction of glycans undergo this cycle (Ganan et al., 1991). Such considerations are especially relevant to the production of Tg in *cog/cog* thyrocytes, which is misfolded (Fig. 6) yet undergoes N-linked glycosylation and glucose trimming comparable to normal Tg (Fig. 2). Interestingly, Tg in the mutant showed no augmentation of calnexin association compared with normal thyrocytes (Fig. 9 A). Thus, although this membrane protein may serve as an important cotranslational chaperone (Chen et al., 1995; Kim and Arvan, 1995), the data does not suggest that calnexin plays a major role in the posttranslational ER retention/quality control of Tg in mutant thyrocytes.

A second function ascribed to ER chaperones is in promoting proper folding of exportable proteins, as opposed to retention/quality control (Hendershot, 1990; Kim et al., 1992). Based on this view, the foregoing results raised the possibility that due to a subtle primary sequence change (such as that causing absence of one especially favored N-linked oligosaccharide [Ware et al., 1995]), abnormal Tg folding might be owed to an inability of Tg to exhibit augmented binding to calnexin. This seemed unlikely, because in three independent studies of the zero chase time, the fraction of newly synthesized Tg bound to calnexin in the mutant was essentially the same as that seen for the normal (data not shown). Nevertheless, to test whether Tg in the mutant might be intrinsically deficient in its ability to form posttranslational complexes with calnexin, we examined this question after exposure of thyrocytes to dithiothreitol, since such binding is maximal after *in vivo* reduction of the ER lumen (Kim and Arvan, 1995). Under these conditions, newly synthesized Tg in normal thyroid was near-quantitatively associated with calnexin (left, Fig. 9 B). This was also true in the mutant (right, Fig. 9 B), suggesting that calnexin and Tg remained compatible binding partners.

Finally, we examined the interaction of newly synthesized Tg with ERp72 (Fig. 9 A, bottom panels). In the normal thyroid, immunoprecipitation of ERp72 was found to coprecipitate relatively little newly-synthesized Tg at any chase time. However, the coprecipitation shown must be regarded as an underestimate of the level of interaction, since this antibody exhibits a diminished immunoprecipitation efficiency under the nondenaturing conditions used. Despite this, a remarkable increase in specific coprecipitation of Tg, exclusively in the mutant, was observed as a function of chase time. Correlating with the Tg folding kinetics by 2D-PAGE (Fig. 6) these data suggested the possibility that misfolded Tg may accumulate in abnormal, ERp72-containing complexes after a delay. Thus, based on these binding studies (Fig. 9) and previous work (Melnick et al., 1994; Kim and Arvan, 1995), at least these four ER

chaperones may associate with secreted proteins, in both simultaneous and sequential manner, and are likely to play unique, albeit possibly overlapping roles in quality control and protein degradation in the ER.

Discussion

ERSDs (Callea et al., 1992) affect numerous organ systems, and represent a large group of hereditary and acquired diatheses which include subsets of patients with alpha-1-antitrypsin deficiency (Sifers et al., 1992), osteogenesis imperfecta (Bonadio and Byers, 1985; Prockop et al., 1985), diabetes insipidus (Schmale et al., 1993), a range of bleeding and clotting disorders (Miura and Aoki, 1990; Miura et al., 1993; Tokunaga et al., 1995), and familial hypercholesterolemia as well as others (for review see Amara et al., 1992). It is currently thought that ERSDs are primarily protein folding disorders. In each instance, the affected gene products are deficient in protein trafficking and accumulate in the ER, where they tend to be degraded (Bonifacino and Lippincott-Schwartz, 1991). Thus, these illnesses provide unique opportunities to study how quality control and ER degradation is regulated by ER chaperones, which may have evolved to protect cells from potentially toxic accumulation of misfolded proteins in the secretory pathway.

In this study, we have examined thyrocytes of homozygous *cog/cog* mice, which like their human counterpart (Michel-Bechet et al., 1969; Lissitzky et al., 1975; Ohyama et al., 1994) exhibit a grossly distended ER (Beamer et al., 1987; Mayerhofer et al., 1988); such cases are new members of the growing family of ERSDs. In *cog/cog* mice, the ERSD is based on ongoing synthesis of Tg molecules that are largely unable to achieve stable homodimers (Fig. 6). Nevertheless, mutant mice synthesize a full-length intact Tg polypeptide that displays normal N-linked glycosylation and glucose trimming (Fig. 2); these processing activities occur during or shortly after protein translocation into the ER of thyrocytes (Franc et al., 1990) as in other higher eukaryotic cells (Gilmore, 1993). However, there is diminished secretion of labeled Tg from mutant thyrocytes (Fig. 3) and near-quantitative accumulation of new Tg in the ER, as confirmed by lack of endo H resistance or endoproteolytic processing (Figs. 4 and 5).

At reduced temperature, an increased fraction of Tg does undergo transport through the secretory pathway (Fig. 7), consistent with a temperature-sensitive folding defect for mutant Tg. Interestingly, even at body temperature, Tg serves as the substrate for synthesis of thyroid hormone in mutant mice (Basche et al., 1989; Adkison et al., 1990; Fogelfeld et al., 1992) supporting the idea that the folding defect for Tg is not maximally severe. Indeed, it is possible that for Tg, like for the $\Delta F508$ -CFTR mutation leading to cystic fibrosis (another ERSD), despite binding to molecular chaperones (Yang et al., 1993; Pind et al., 1994), a meaningful fraction of ER-entrapped molecules could be functionally competent (Pasyk and Foskett, 1995). Thus, an important contribution to the ERSD phenotype may derive from the quality control machinery itself, the stringency of which limits release of exportable proteins that have potential biological activity.

For this reason, we have attempted to examine known components of this machinery, comprised primarily of ER chaperones. By comparative immunoblotting of wild-type and mutant thyrocytes, cellular levels of PDI, calnexin, and ribophorin I are on average ~ 2.3 -fold elevated in the mutant cells, consistent with a generalized increase in size of the ER compartment (Wiest et al., 1990; Rajasekaran et al., 1993). Notably, the relative concentration of PDI in the ER is not further increased, consistent with previous reports (Macer and Koch, 1988; Dorner et al., 1990). By contrast, five ER luminal chaperones are induced in the mutant cells by an average of approximately eightfold, suggesting an $>300\%$ increase in their relative ER concentration (Fig. 8). Despite that these are already among the most abundant proteins contained in the normal ER (Marquardt et al., 1993), they appear to dominate the entire mutant thyroid gland (Adkison et al., 1990; and our unpublished observations). We conclude that these particular five proteins are central to the compensatory response to this ERSD.

One might surmise that the induction of these five chaperones, by itself, increases the efficiency of folding and assembly of mutant Tg and thereby brings about a "self-cure". This seems highly unlikely for several reasons. First, although helper function of ER chaperones is certainly possible (Kim et al., 1992; Hammond and Helenius, 1994) we find that even in chaperone-induced mutant thyrocytes, Tg export efficiency falls at or below the limits of detection (a few percent) in our assays. Second, in the lifespan of *cog/cog* mice, the induction of chaperones greatly precedes the correction of biochemical insufficiency of thyroid hormone (our unpublished data). Third, little evidence currently exists to directly demonstrate that ER chaperones actually assist (except for the catalytic activity of PDI [Weissman and Kim, 1993]) in folding of exportable proteins (Kim et al., 1992; Hammond and Helenius, 1994), or that elevated levels of ER chaperones can improve folding or transport efficiency (Dorner et al., 1992). In fact, the major physiological response that overcomes the genetic defect during the natural history of this illness is the increased pituitary secretion of thyroid stimulating hormone that causes massive hypertrophy and hyperplasia of the thyroid gland (i.e., goiter). Obviously, if the efficiency of Tg secretion were only 5% of wild-type levels, a >20 -fold increase in Tg production by an expanded goiter could largely correct the problem; moreover, the kinetics of growth of the thyroid gland actually parallels or even precedes the recovery of thyroid hormone levels in the bloodstream of untreated mutant animals (Adkison et al., 1990).

Lowering "free BiP" as a consequence of BiP binding induces BiP synthesis (Kohno et al., 1993; Mori et al., 1993). From the specific increase in concentrations of other ER chaperones, one might be tempted to infer that each of these chaperones also physically interacts with misfolded Tg. However, while overexpression of individual ER chaperones may not suppress synthesis of others, the promoter regions of these five chaperones do exhibit common elements, suggesting that group regulation of chaperone synthesis occurs (Dorner et al., 1990; McCauliffe et al., 1992; Srinivasan et al., 1993). Thus, it is still necessary to establish the presence of direct interactions of Tg with individ-

ual chaperones using biochemical means (Kim et al., 1992; Kim and Arvan, 1993, 1995).

In the normal mouse, the interaction of newly synthesized Tg with BiP exhibits dissociation kinetics (Fig. 9) consistent with a folding pathway for Tg similar to that reported in porcine cells (Kim et al., 1992; Kim and Arvan, 1993). The interaction of normal Tg with, and dissociation from GRP94 and BiP cannot be resolved by kinetic criteria (Fig. 9), although we have not yet determined if these represent ternary, individual, or even sequential interactions (Melnick et al., 1994). Nevertheless, in the mutant, the kinetics of these interactions changed such that associations with both chaperones were dramatically prolonged (Fig. 9). Thus, while it has been shown that both BiP and GRP94 can physically interact with Tg (Kuznetsov et al., 1994), the present data represent the first demonstration that both hsp 70 and hsp 90 family members participate significantly in ER retention/quality control of Tg export.

By contrast, calnexin, whose interaction with Tg has been proposed to occur largely cotranslationally rather than posttranslationally (Kim and Arvan, 1995), showed no increased binding to Tg in the mutant vs the normal (Fig. 9), lending no support to the idea that this ER membrane chaperone plays a significant role in posttranslational ER retention/quality control for Tg. Several factors may account for this. First, in agreement with our quantitative immunoblotting (Fig. 8), the synthesis of calnexin has not been found to be especially affected by the unfolded protein response (Bergeron et al., 1994). Thus, it is expected that, in the face of increasing relative concentrations of luminal chaperones, the competition for binding sites would not favor enhanced calnexin association. Second, surface-to-volume considerations (based on the geometry of the ER compartment) suggest that calnexin might not be an ideal retention factor for proteins contained largely in the ER lumen. Third, it is not clear that the particular structural information that calnexin binds during Tg entrance into the ER is different in normal and mutant thyroids. In this regard, despite that calnexin has been reported to function as a lectin (Ware et al., 1995) whose interaction is disrupted by inhibition of ER glucosidases (Hebert et al., 1995), we have found that the interaction of calnexin with newly synthesized Tg is essentially independent of N-glycosylation (Kim and Arvan, 1995); moreover, castanospermine treatment (Fig. 2) has no effect on the coprecipitation of newly synthesized Tg with calnexin in either normal or mutant mouse thyroids (our unpublished data). Fourth, recent evidence has suggested that calreticulin may have lectinlike properties (Wada et al., 1995; Peterson et al., 1995); unlike calnexin, calreticulin is induced significantly in the mutant thyroid (Fig. 8). Thus, while we do not favor a lectin interaction of calnexin with Tg in the posttranslational period, such an interaction for calreticulin is by no means excluded.

Since accumulation of mutant Tg in the ER is not a lethal condition for thyrocytes, the large fraction of Tg that fails to be exported must be degraded (Bonifacino and Lippincott-Schwartz, 1991). At present, little or nothing is known about how Tg is degraded in the mutant thyroid. However, as a clue to this process, we note that the distribution of pulse-labeled Tg shifts by native gel electrophoresis to a position of diminished mobility upon chase

(Fig. 6). Although the molecular basis for this shift has yet to be determined, such behavior is compatible with the formation of novel Tg-containing complexes en route to degradation. With this in mind, we note that exclusively in the mutant, as a function of chase time (Fig. 9), there is increased interaction of Tg with ERp72, one of the proteins selectively induced in the thyroids of mutant mice (Fig. 8). Indeed, these association properties are consistent with possible involvement of ERp72 in degrading misfolded proteins or targeting such proteins for ER degradation (Urade et al., 1993) along with ER60, another luminal protein (Otsu et al., 1995) that is selectively induced in the thyroids of mutant mice (Fig. 8). Finally, we note that *cog/cog* thyrocytes accumulate reasonable quantities of a ~45 kD NH₂-terminal Tg fragment that is not seen in normal thyrocytes (Fig. 5), which may represent a partial proteolytic product from the ER degradation process. Further work will be needed to characterize the degree to which each ER chaperone influences the fate of Tg and other secretory proteins in normal cells and in ERSDs.

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