Folding of thyroglobulin in the calnexin/calreticulin pathway and its alteration by loss of Ca^{2+} from the endoplasmic reticulum

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During its initial folding in the endoplasmic reticulum (ER), newly synthesized thyroglobulin (Tg) is known to interact with calnexin and other ER molecular chaperones, but its interaction with calreticulin has not been examined previously. In the present study, we have investigated the interactions of endogenous Tg with calreticulin and with several other ER chaperones. We find that, in FRTL-5 and PC-Cl3 cells, calnexin and calreticulin interact with newly synthesized Tg in a carbohydrate-dependent manner, with largely overlapping kinetics that are concomitant with the maturation of Tg intrachain disulphide bonds, preceding Tg dimerization and exit from the ER. Calreticulin co-precipitates more newly synthesized Tg than does calnexin; however, using two different experimental approaches, calnexin and calreticulin were found in ternary complexes with Tg, making this the first endogenous protein reported in ternary complexes with calnexin and calreticulin in the ER of live cells. Depletion of Ca^{2+} from the

ER elicited by thapsigargin (a specific inhibitor of ER Ca^{2+} -ATPases) results in retention of Tg in this organelle. Interestingly, thapsigargin treatment induces the premature exit of Tg from the calnexin/calreticulin cycle, while stabilizing and prolonging interactions of Tg with BiP (immunoglobulin heavy chain binding protein) and GRP94 (glucose-regulated protein 94), two chaperones whose binding is not carbohydrate-dependent. Our results suggest that calnexin and calreticulin, acting in ternary complexes with a large glycoprotein substrate such as Tg, might be engaged in the folding of distinct domains, and indicate that luminal Ca^{2+} strongly influences the folding of exportable glycoproteins, in part by regulating the balance of substrate binding to different molecular chaperone systems within the ER.

Key words: calreticulin, chaperone, endoplasmic reticulum microenvironment, folding, glycoprotein, thyroglobulin.

INTRODUCTION

The endoplasmic reticulum (ER) is the site of synthesis of membrane and secretory proteins. As they enter the lumen of the ER during the translocation process, newly synthesized membrane and secretory proteins are subjected to the action of enzymes and molecular chaperones that assist their conformational maturation. Among the chaperones of the ER are two related lectin-type chaperones, calnexin and calreticulin. Calnexin is a type I membrane protein which has been demonstrated to bind transiently to several glycoproteins via partially trimmed N-linked oligosaccharide chains carrying a single glucose residue [1–3]. The association of calnexin with its substrate glycoproteins facilitates their folding and oligomerization and supports quality control [4,5]. More recently, numerous proteins have been found to associate not only with calnexin but also with calreticulin, a soluble protein of the ER that shares extensive sequence similarity with the luminal domain of calnexin [6–10]. Calreticulin is believed to possess chaperone and quality-control functions similar to those of calnexin [11,12].

Calnexin and calreticulin possess identical oligosaccharide binding specificity [6,13,14] and there is an overlap in the glycoproteins that they bind [15]. Recent data, however, indicate

that the bound proteins observed following co-immunoprecipitation from pulse-labelled cells are not identical; therefore some glycoproteins may prefer calnexin, while others prefer calreticulin, for their folding, perhaps because of topological constraints [6,16]. It has been suggested that calreticulin may have a preference for secretory proteins, while calnexin prefers membrane proteins [6]. In accordance with this model, vesicular stomatitis virus G (VSV-G) protein binds to calnexin, but not to calreticulin [2,6]. In addition, a sequential mode of interaction of calnexin and calreticulin with certain substrate proteins has been documented experimentally. In fact, calreticulin dissociates more rapidly than does calnexin from the T-cell receptor [7] and from influenza virus haemagglutinin (HA) [17] as their folding proceeds, and the two chaperones bind to MHC class I antigens at different stages of maturation [18].

Notably, despite the very large number of glycoproteins that have been shown to interact with calnexin and calreticulin, ternary complexes of protein substrate, calnexin and calreticulin have been demonstrated in only a few instances, i.e. for HIV gp 160 (type I envelope glycoprotein) [19] and for HA *in vitro* (but not *in vivo*) [6,17]. Therefore the occurrence of substrate–calnexin–calreticulin ternary complexes has yet to be demonstrated for an endogenous protein in the ER of live cells.

Abbreviations used: BiP, immunoglobulin heavy chain binding protein; CST, castanospermine; dNM, 1-deoxynojirimycin; DSP, dithiobis(succinimidylpropionate); endoH, endo-N-acetylglucosaminidase; ER, endoplasmic reticulum; GRP94, glucose-regulated protein 94; HA, influenza virus haemagglutinin; PNGase F, peptide N-glycosylhydrolase F; Tg, thyroglobulin; VSV-G, vesicular stomatitis virus G protein.

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However, such a possibility seems reasonable for proteins above a certain molecular size that may have distinct domains whose folding depends upon the action of calnexin or calreticulin, or both. Therefore, in addition to proteins that show selectivity for calnexin or calreticulin and proteins that bind sequentially to calnexin and calreticulin, there may exist proteins that bind simultaneously to both lectin-type chaperones.

Thyroglobulin (Tg), the molecule used for the synthesis and storage of thyroid hormones, is a very large glycoprotein: it is a homodimer of 660 kDa with up to 60 disulphide bonds and between 10 and 15 N-linked oligosaccharides per Tg monomer (depending on the species) [20]. The folding of Tg is extremely slow: the $t_{1/2}$ for arrival in the medial Golgi in primary porcine thyrocytes is 2 h [21], and folding involves calnexin, BiP (immunoglobulin heavy chain binding protein) [22], GRP94 (glucose-regulated protein 94) and ERp72 [23,24]. Because of its large number of N-linked oligosaccharides and its secretory nature, it seems unlikely that the folding of Tg depends on calnexin as the only lectin-type chaperone (since calnexin may prefer membrane glycoprotein substrates) [6], although an interaction of Tg with calreticulin has not yet been reported. Importantly, however, it has been reported that treatment of thyroid cells with castanospermine (CST; an ER glucosidase inhibitor that prevents the binding of glycoproteins to calnexin/calreticulin) [2,6] delays Tg export [25], suggesting a functional role for calnexin and calreticulin in the conformational maturation of Tg. For these reasons, we decided to study specifically the interactions of newly synthesized Tg with both lectin-type chaperones calnexin and calreticulin.

We have found that Tg interacts with both calnexin and calreticulin, with similar kinetics. This represents the first example of an endogenous protein that has been demonstrated to be present in ternary complexes with these chaperones in the ER of live cells. Moreover, loss of Ca^{2+} from the ER induced by thapsigargin causes the retention of Tg in the ER [26] and activates the unfolded protein response [27], suggesting defective folding of Tg in the ER lumen. Consistent with this, we demonstrate that thapsigargin causes premature exit of Tg from the calnexin/calreticulin cycle, simultaneous with stabilization and prolongation of Tg binding to BiP and GRP94.

EXPERIMENTAL

Materials

Coon's modified Ham's F-12 medium was from Sigma. Protein A-Sepharose was from Calbiochem. Endo-*N*-acetylglucosaminidase (endoH) was from Boehringer Mannheim. [^{35}S]-Cysteine/[^{35}S]-methionine Promix was from Amersham. All other chemicals were from Sigma.

Cell culture and biological reagents

FRTL-5 cells (A.T.C.C. CRL 8305) are a continuous, cloned line of thyroid differentiated cells [27a]. These cells were maintained as described in [28]. Polyclonal anti-Tg rabbit antibodies were raised against rat Tg as described previously [28]. Anti-calnexin antibodies were from Stressgen, and anti-calreticulin, anti-BiP and anti-GRP94 antibodies were from Affinity Bioreagents.

Metabolic labelling and immunoprecipitation

FRTL-5 cells were incubated for 30 min in methionine/cysteine-free medium, then labelled for 5 min at 37 °C in medium without unlabelled cysteine and methionine but containing [^{35}S]-cysteine and [^{35}S]-methionine (50 μCi of L-[^{35}S] *in vitro* cell labelling mix/ml) and chased either in a normal medium with an excess of

Figure 1 Newly synthesized Tg associates with calnexin and calreticulin

FRTL-5 cells were labelled as outlined in the Experimental section and chased for 15 min. Equal amounts of cell lysates were subjected to immunoprecipitation with non-immune serum (CON; lane 1), anti-calnexin antibodies (lane 2), anti-calreticulin antibodies (lane 3) or anti-Tg antibodies (lane 4) under non-denaturing conditions (A). In (B), four times larger amounts of cell lysates were immunoprecipitated with non-immune serum (lane 1), anti-Tg antibodies (lane 2), anti-calnexin antibodies (lane 3) and anti-calreticulin antibodies (lane 4) under denaturing conditions. Immunoprecipitated proteins were visualized by SDS/PAGE and fluorography. The positions of Tg, calnexin (CNX), calreticulin (CRT) and molecular-mass markers (kDa) are indicated.

unlabelled methionine and cysteine or, for the experiments employing thapsigargin, in a nominally Ca^{2+} -free medium with the same excess of unlabelled amino acids. All subsequent manipulations were performed at 4 °C. The labelled cells were washed with cold PBS containing 20 mM *N*-ethylmaleimide in order to alkylate free thiols and then lysed in 1 ml of 0.5% Triton X-100 lysis buffer [25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 20 mM *N*-ethylmaleimide and 'complete' protease inhibitor mixture (Boehringer Mannheim)]. Nuclear and cellular debris were removed by centrifugation at 10000 *g* for 10 min at 4 °C. In the experiments depicted in Figures 1(A), 2(B) and 3, lysates were precleared with Protein A-Sepharose without antibodies. The lysates were incubated for 1 h at 4 °C with the respective antibodies and tumbled with Protein A-Sepharose for 3 h. The beads were washed three times with lysis buffer, boiled in SDS sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 20 mM dithiothreitol, 0.2% Bromophenol Blue and 20% glycerol), and the supernatant was subjected to SDS/PAGE on 7.5% (w/v) acrylamide gels (unless indicated otherwise) and autoradiography.

For BiP immunoprecipitations, labelled cells were washed with cold PBS containing 20 mM *N*-ethylmaleimide and then three times with PBS alone, and lysed in 1 ml of 0.5% Triton X-100 lysis buffer [25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 'complete' protease inhibitor mixture and 20 units/ml apyrase (to enzymically deplete ATP)]. After 60 min, cells were processed as above.

Chemical cross-linking was performed as reported previously with minor modifications [24]. Briefly, labelled cells were rinsed with PBS and then incubated for 30 min at 4 °C with 2 mM dithiobis(succinimidylpropionate) (DSP) in PBS (from a stock 20 mM solution in DMSO, freshly made for each experiment). For control samples an equivalent amount of DMSO without DSP was added. Cells were rinsed with cold PBS and then alkylated with cold PBS containing 20 mM *N*-ethylmaleimide.

Figure 2 Trimming by glucosidases I and II is required for the initial association of Tg with calnexin and calreticulin

FRTL-5 cells were preincubated with CST (CAST) (**A**) or CST plus dNM (**B**) for 1 h, then labelled as outlined in the Experimental section and chased for 15 min. In (**A**), cell lysates were immunoprecipitated with anti-Tg antibodies (Ab), and the immunoprecipitates were divided in two and digested with endoH or mock-digested. Immunoprecipitates were resolved by SDS/PAGE (5% acrylamide gels). In (**B**), equal amounts of cell lysates were immunoprecipitated with non-immune serum (CON; lanes 1, 5 and 9), anti-Tg antibodies (lanes 2, 6 and 10), anti-calnexin antibodies (lanes 3, 7 and 11) or anti-calreticulin antibodies (lanes 4, 8 and 12). Immunoprecipitates were resolved by SDS/7.5%-PAGE. The positions of Tg, calnexin (CNX), calreticulin (CRT) and molecular-mass markers (kDa) are indicated.

Figure 3 Tg associates with calnexin and calreticulin during the same period

FRTL-5 cells were labelled as outlined in the Experimental section and chased for the indicated times. Equal amounts of cell lysates were subjected to immunoprecipitation with non-immune serum (lanes 1–5), anti-calnexin antibodies (Ab) (lanes 6–10), anti-calreticulin antibodies (lanes 11–15) or anti-Tg antibodies (lanes 16–20). Immunoprecipitates were resolved by SDS/PAGE. The positions of Tg, calnexin (CNX), calreticulin (CRT) and molecular-mass markers (kDa) are indicated.

Cells were lysed with 1 ml of 0.5% Triton X-100 lysis buffer (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 20 mM *N*-ethylmaleimide and 'complete' protease inhibitor mixture) containing 50 mM glycine to quench the excess cross-linker. Lysates were processed as above.

For immunoprecipitation under denaturing conditions, cells were labelled, washed and lysed as above. One-quarter of the lysate was immunoprecipitated as above, and the remainder was brought to 5% (v/v) SDS and boiled for 5 min. The SDS was quenched by the addition of 10 vol. of 1% Triton X-100 in 25 mM Tris/HCl (pH 7.4)/150 mM NaCl, and immunoprecipitated with non-immune serum or anti-Tg, anti-calnexin and anti-calreticulin antibodies. Non-denatured samples were washed as above, and denatured samples were washed three times with 0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl and 10 mM Tris/HCl, pH 8.6.

Quantification was performed using a PhosphorImager (Molecular Dynamics). Stock and diluted solutions of the drugs were made in DMSO. Generally, a 2 μ l volume of the drug was added to 1 ml of culture medium. The controls received pure DMSO.

Sucrose gradient centrifugation

Cells were labelled and washed as outlined above. Cells were lysed with 0.5 ml of 0.15 M Tris, pH 7.5, 300 mM NaCl, 1% Triton X-100, 20 mM *N*-ethylmaleimide and protease inhibitors. In some experiments, in order to lyse the cells gently, Triton X-100 was replaced with a mixture of 1% Lubrol PX and egg phosphatidylcholine at a molar ratio of 1:7 [29]. Cell lysates were incubated for 30 min on ice. After centrifugation at 10000 *g* for 30 min at 4 °C, supernatants were layered on top of a continuous sucrose gradient (5–28%, w/v) in 0.15 M Tris, pH 7.5, 300 mM NaCl and 0.1% Triton X-100. Centrifugation was for 18 h at 20 °C in a Beckman SW41 rotor at 26000 rev./min. Fraction size was 0.25 ml. Iodine-labelled Tgs [bovine (dimers) or from guinea pigs], treated with propylthiouracil for at least 4 weeks (dimers and monomers), served as standard proteins.

Other methods

Digestions with endoH and peptide *N*-glycosylase F (PNGase) were performed as reported previously [26,28].

RESULTS

Newly synthesized Tg associates with calnexin and calreticulin

We looked for an interaction of Tg with calreticulin using as a model system FRTL-5 cells, a fully differentiated rat thyroid cell line. FRTL-5 cells were radiolabelled for 5 min with [³⁵S]-methionine and chased for 15 min. Cell lysates were immunoprecipitated with non-immune serum or with anti-calnexin, anti-calreticulin or anti-Tg antibodies, and analysed by SDS/PAGE and autoradiography. We looked for co-precipitation of Tg by anti-calnexin and anti-calreticulin antibodies. When the lysates were precipitated with anti-calnexin antibodies, a labelled calnexin band at approx. 90 kDa could be observed (Figure 1A, lane 2). In addition, a band corresponding to Tg was present at 330 kDa (Figure 1A, lane 2). When the lysates were immunoprecipitated with anti-calreticulin antibodies, a labelled calreticulin band of approx. 60 kDa [30] was present. Again a Tg band could be seen (Figure 1A, lane 3). For comparison, lysates were immunoprecipitated with anti-Tg antibodies directly (Figure 1A, lane 4). Quantification of the radioactivity in the bands revealed that 68% of newly synthesized Tg was associated with calreticulin and 39% with calnexin. These immunoprecipitates were also solubilized in SDS-containing buffer, and re-immunoprecipitated with anti-Tg antibodies. Quantification of the re-immunoprecipitated Tg gave identical results (results not shown).

In these co-immunoprecipitation experiments, other bands in addition to Tg were present, although Tg represented the great majority of the co-immunoprecipitated material. A few of these bands were aspecific, since they were also visible in the non-immune lane. Other bands probably represented cellular proteins associated with calnexin and calreticulin, since they were absent from the non-immune lane and were chased away in pulse-chase experiments (see Figure 3, lanes 6–15). The immunoprecipitations shown in Figure 1(A), and the others throughout the study, were quantitative, since a second round of immunoprecipitation did not give any co-immunoprecipitated Tg. To confirm the specificity of the anti-calnexin and anti-calreticulin antibodies, immunoprecipitations were carried out under denaturing conditions. Under these conditions, the anti-calnexin and anti-calreticulin antibodies immunoprecipitated calnexin and calreticulin only (Figure 1B, lanes 3 and 4). The experiment in Figure 1(B) also excluded the possibility of any cross-reactivity of the anti-calnexin antibodies with calreticulin, and vice versa. To further rule out any cross-reactivity of the anti-calnexin and anti-calreticulin antibodies with Tg, we took advantage of the Tg secreted into the medium by FRTL-5 cells, which is clearly not associated with calnexin and calreticulin. Anti-calnexin and anti-calreticulin antibodies were totally unable to immunoprecipitate the secreted Tg under non-denaturing conditions (results not shown).

Experiments identical with those shown in Figure 1 were also carried out in another fully differentiated thyroid cell line, PC-C13 cells, with the same results (not shown). We also performed immunoprecipitations using the protocol of Helenius and co-workers [2,3] (lysis in 2% CHAPS, washing in 0.5% CHAPS) without observing appreciable differences. Therefore we concluded that the great majority of newly synthesized Tg was associated with calnexin and calreticulin.

In order to assess whether glucose trimming was a prerequisite for the binding of newly synthesized Tg to calnexin and

Figure 4 Unfolded Tg binds to calnexin and calreticulin: association of Tg with calnexin and calreticulin precedes dimerization and export to the Golgi complex

(A) FRTL-5 cells were labelled as outlined in the Experimental section and chased for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-Tg antibodies and resolved by SDS/5%-PAGE under non-reducing (NR) and reducing (R) conditions. (B) Kinetics of dissociation of Tg from calnexin/calreticulin were calculated from three separate experiments similar to that shown in Figure 3 (◇, dissociation from calreticulin; ■, dissociation from calnexin). For evaluation of Tg dimerization, FRTL-5 cells were labelled as outlined in the Experimental section and chased for the indicated times. Cell lysates were loaded on continuous sucrose gradients. Tg was immunoprecipitated from individual fractions and resolved by SDS/PAGE and autoradiography. The amount of dimeric Tg is plotted against the time of chase (△). Arrival at the medial-Golgi was evaluated by monitoring the acquisition of resistance to endoH by newly synthesized Tg. FRTL-5 cells were labelled and chased for the indicated times. Cells were lysed and Tg was immunoprecipitated. The immunoprecipitates were divided in two, digested with endoH or mock-digested, and resolved by SDS/5%-PAGE. The appearance of a slow-migrating, endoH-resistant-Tg band was quantified by phosphorimaging. The amount of endoH-resistant Tg is plotted against the time of chase (○). To evaluate Tg secretion, FRTL-5 cells were labelled and chased for the indicated times. Secreted and intracellular Tg were immunoprecipitated, resolved by SDS/PAGE and quantified by phosphorimaging. The amount of labelled secreted Tg is plotted against the time of chase (■). In all cases, the mean ± S.D. of at least three separate experiments is shown.

calreticulin, we used two α -glucosidase inhibitors, CST and 1-deoxynojirimycin (dNM), which inhibit glucosidases I and II. First, we wanted to set up experimental conditions that allowed inhibition of glucosidases I and II. Therefore FRTL-5 cells were incubated for 1 h with 0.1 mg/ml CST, radiolabelled for 5 min with [³⁵S]methionine and chased for 15 min. Tg was immunoprecipitated, digested with PNGase F or mock-digested, and resolved on SDS/PAGE. Figure 2(A) shows that, following CST treatment, Tg migrated slightly more slowly than Tg from control cells. Moreover, digestion with PNGase F, which removes all N-linked carbohydrate chains from glycoproteins, abolished the different migration. We concluded that CST treatment was effective in preventing the removal of one or more glucose residues from N-linked carbohydrate chains. Thus FRTL-5 cells were preincubated with CST or dNM or mock-incubated for 1 h, then radiolabelled for 5 min with [³⁵S]methionine and chased for 15 min. Cell lysates were immunoprecipitated with non-immune serum or anti-calnexin, anti-calreticulin or anti-Tg antibodies, and resolved by SDS/PAGE and autoradiography. Figure 2(B) shows that, whereas the amounts of calnexin and calreticulin were unaffected by CST and dNM, the association of newly synthesized Tg with these chaperones was abolished by CST and

Figure 5 Tg forms ternary complexes with calnexin and calreticulin

Upper panel: FRTL-5 cells routinely grown as outlined in the Experimental section were lysed and immunoprecipitated (IP) with anti-Tg, anti-calnexin or anti-calreticulin antibodies or non-immune serum covalently coupled to CNBr–Sepharose. The immunoprecipitates were resolved by SDS/PAGE and subjected to Western blotting (WB) using anti-Tg, anti-calnexin or anti-calreticulin antibodies. Lower panel: FRTL-5 cells were labelled and chased for 10 min. The cells were lysed, and the extract was divided in four and immunoprecipitated with anti-calnexin (1° Ab calnexin; lanes 1–4), anti-calreticulin (1° Ab calreticulin; lanes 5–8) or anti-oestrogen receptor (1° Ab EsR; lanes 9–16) antibodies. To completely deplete calnexin- or calreticulin-bound Tg from the cell extract, the supernatants from the first immunoprecipitation were re-immunoprecipitated with the same respective antibodies (anti-calnexin, lane 2; anti-calreticulin, lane 6; anti-EsR, lanes 10 and 14). Successively, to remove any free calnexin- and calreticulin-directed antibodies from these supernatants, an excess of Protein A–Sepharose was added to each supernatant (lanes 3, 7, 11 and 15). Finally, the supernatant depleted of calnexin was used for immunoprecipitation by anti-calreticulin antibodies (lane 4), the supernatant cleared from calreticulin was used for immunoprecipitation by anti-calnexin antibodies (lane 8), and the supernatants not cleared were used for immunoprecipitation by anti-calreticulin (lane 12) and anti-calnexin (lane 16) antibodies. Proteins associated with the Protein A–Sepharose at each step were analysed by SDS/PAGE and autoradiography. The positions of Tg, calnexin (CNX), calreticulin (CRT) and molecular-mass markers (kDa) are indicated.

strongly inhibited by dNM. Less efficient inhibition elicited by dNM compared with CST of the binding of glycoproteins to calnexin and calreticulin has been reported previously [3], suggesting that dNM allows one or two glucoses to be removed. As a control, we used 1-deoxymannojirimycin, an inhibitor of α -

Figure 6 Thapsigargin causes premature exit of Tg from the calnexin/calreticulin cycle

FRTL-5 cells were labelled as outlined in the Experimental section and chased in a nominally Ca²⁺-free medium in the presence or in the absence of 5 μ M thapsigargin (THAPS) for the indicated times. Two-thirds of the cell lysate was subjected to immunoprecipitation with anti-calnexin antibodies (Ab) (lanes 1–9), while one-third was immunoprecipitated with anti-calreticulin antibodies (lanes 10–18) in order to obtain comparable Tg band intensities and directly compare the dissociation kinetics in absence and presence of thapsigargin. Immunoprecipitates were resolved by SDS/PAGE. The positions of Tg, calnexin (CNX), calreticulin (CRT) and molecular-mass markers (kDa) are indicated.

mannosidase, which was without effect on the association of calnexin and calreticulin with Tg (results not shown). These results indicate that the initial association of Tg with both calnexin and calreticulin depends on the glucose trimming of N-linked carbohydrate chains, as shown for a number of glycoproteins [15].

Comparison of the kinetics of binding of Tg to calnexin and calreticulin

To evaluate the timing of Tg binding to calnexin and calreticulin, pulse-chase experiments were performed. FRTL-5 cells were pulse-labelled for 5 min and chased for 0–90 min. Cell lysates were immunoprecipitated with non-immune serum or anti-calnexin, anti-calreticulin or anti-Tg antibodies, and resolved on SDS/PAGE (Figure 3). In the anti-Tg immunoprecipitates it appeared that the amount of radioactivity in the Tg band increased dramatically during the first 10 min of chase (Figure 3, lanes 16 and 17). This effect, which has been reported by several authors, was caused by completion of nascent Tg chains labelled but not terminated during the pulse [31]. At 90 min of chase, Tg band intensity was clearly decreased, since secretion had already begun (Figure 3, lane 20). Quantification of the bands revealed that, for the time points explored, Tg associated maximally with calnexin and calreticulin at 10 min of chase (approx. 35% of newly synthesized Tg associated with calnexin, and approx. 65% with calreticulin) (Figure 3, lanes 7 and 12, and Figure 4B). We found $t_{1/2}$ values for dissociation of 45 min for calnexin and of 38 min for calreticulin. Therefore calnexin and calreticulin associate with Tg over the same time period (Figure 4B). In addition to Tg, the pattern of other bands associated with calnexin and calreticulin was extremely reproducible in all experiments throughout this study (see electrophoretic patterns of Figures 1A, 2B, 3, 5B and 6). This fact, together with the

absence of these other bands from the non-immune lanes and their dissociation during the chase, strongly suggests that they represent other cellular proteins specifically associated with calnexin and calreticulin.

Unfolded Tg binds to calnexin and calreticulin: the association of Tg with calnexin and calreticulin precedes dimerization and export to the Golgi complex

The existence of transient Tg–calnexin and Tg–calreticulin complexes prompted us to investigate the temporal relationship between the dissociation of Tg from these chaperones, Tg conformational maturation, Tg dimerization [21] and arrival of Tg in the medial-Golgi compartment. To evaluate the conformational maturation of Tg, Tg immunoprecipitates of pulse-labelled and chased cells were resolved under non-reducing and reducing conditions. Under non-reducing conditions, the newly synthesized Tg band appeared broad and, during the chase, the electrophoretic mobility of Tg increased up to 60 min (Figure 4A, lanes 1–4). At 90 min of chase the mobility had not increased and its intensity had decreased, since secretion had already begun (Figure 4A, lane 5). Under reducing conditions there was no change in the mobility of Tg, which migrated as a single sharp band of identical mobility at all times of chase, indicating that the mobility differences observed on non-reducing gels are due to the maturation of intrachain disulphide bonds in nascent Tg. During the period of association of Tg with calnexin and calreticulin, there was no evidence of fully oxidized Tg (which did not appear until 60 min of chase), and a major proportion (if not all) of newly synthesized Tg was associated with calnexin and calreticulin (Figure 3). Therefore we did not analyse calnexin and calreticulin immunoprecipitates under non-reducing conditions, and we concluded that Tg associated with calnexin and calreticulin was unfolded and in the process of undergoing disulphide-bond maturation. We have reproducibly obtained a time-dependent increase in the electrophoretic mobility of Tg under non-reducing conditions in numerous experiments.

Next we evaluated Tg dimerization by sucrose gradient centrifugation. The time course of Tg dimerization is shown in Figure 4(B) (Δ). Dimerization was a slow process, with the first detectable dimers appearing after 30 min of chase. The proportions of Tg sedimenting as monomers and dimers were not altered by the method used to lyse the cells, since lysis with either 1% Triton X-100 or 1% Lubrol PX/phosphatidylcholine, which prevents oligomer dissociation [29], did not change their ratio (results not shown). Next we monitored the acquisition of resistance to endoH as a marker of Tg transport at the medial-Golgi (Figure 4B) ($\circ$). FRTL-5 cells were pulse-labelled and chased for the indicated times. Cells were lysed, and intracellular and secreted Tg was immunoprecipitated, digested or not with endoH and resolved by SDS/PAGE. The first molecules resistant to endoH were detected after 60 min, and their amount increased thereafter (Figure 4B). Figure 4(B) also shows the time course of Tg secretion ($\blacksquare$). A clear temporal sequence of these events emerged. The association of nascent monomeric Tg with calnexin and calreticulin was the first of these processes, occurring immediately after Tg synthesis. This association was transient, and was followed by dissociation of Tg from calnexin/calreticulin, concomitant with isomerization/oxidation of disulphide bonds. These events largely preceded dimerization (e.g. at 90 min, when ~ 50% of Tg molecules were still monomeric, the amount of Tg bound to calnexin and calreticulin had fallen to 14% and 12% respectively of the maximum bound at 10 min). Dimerization, in turn, preceded the arrival of Tg at the medial-Golgi. This is consistent with earlier findings that when Tg dimers and

monomers are subjected separately to digestion by endoH, only Tg dimers display resistance to endoH, with the monomers being endoH-sensitive [26].

Therefore Tg dimerization and transport out of the ER normally occur after dissociation of Tg from calnexin and calreticulin, which occurs synchronously with disulphide bond maturation.

Tg forms ternary complexes with calnexin and calreticulin

Next we tested for the occurrence of ternary complexes of Tg, calnexin and calreticulin in the ER of FRTL-5 cells. As a first approach, we performed Western blot analysis of co-immunoprecipitates with anti-Tg, anti-calnexin and anti-calreticulin antibodies. As expected from the results of the metabolic labelling experiments (Figure 1A, lanes 2 and 3, Figure 2B, lanes 3 and 4, and Figure 3, lanes 6–15), immunoprecipitation with anti-calnexin and anti-calreticulin antibodies and Western blot analysis with anti-Tg antibodies revealed the presence of co-immunoprecipitated Tg (Figure 5, upper panel, lanes 2 and 3). Furthermore, anti-Tg immunoprecipitates probed with anti-calnexin and anti-calreticulin antibodies showed the presence of calnexin and calreticulin (Figure 5, upper panel, lanes 4 and 7). In metabolic labelling experiments, we did not detect calnexin and calreticulin in anti-Tg immunoprecipitates. This is probably because only a very small fraction of total cellular calnexin and calreticulin molecules were labelled; in addition, there may exist a selectivity in the binding of newly synthesized Tg to pre-existing chaperone molecules. Notably, anti-calnexin immunoprecipitates revealed the presence of calreticulin (Figure 5, upper panel, lane 8) and anti-calreticulin immunoprecipitates showed the presence of calnexin (lane 6), suggesting the occurrence of ternary Tg–calnexin–calreticulin complexes.

To substantiate this result further, sequential double immunoprecipitations were performed. FRTL-5 cells were pulse-labelled for 5 min and chased for 15 min. Half of the extract was depleted of calnexin-bound Tg by two successive immunoprecipitations with anti-calnexin antibodies and subsequent incubation with Protein A only, and the other half was depleted of calreticulin-bound Tg in a similar way (Figure 5, lower panel, lanes 1–3 and 5–7 respectively). Finally, the extract depleted of calnexin-bound Tg was immunoprecipitated with anti-calreticulin antibodies, and the extract depleted of calreticulin-bound Tg was immunoprecipitated with anti-calnexin antibodies. It was clear that lysates depleted of calnexin-bound Tg still contained significant amounts of calreticulin-bound Tg, whereas the anti-calreticulin antibodies almost completely depleted calnexin-bound Tg (Figure 5, lower panel, lanes 4 and 8 respectively). These results indicate that the majority of Tg molecules that are bound to calnexin are also bound to calreticulin in ternary complexes, and that there are also Tg–calreticulin binary complexes. Figure 5 (lower panel) also shows that when the first two rounds of immunoprecipitation were performed with unrelated antibodies, raised against the oestrogen receptor, the complexes containing calnexin were still recovered in the last immunoprecipitation, ruling out the possibility that they were dissociated during this procedure. In FRTL-5 cells, Tg was monomeric immediately after synthesis (at the end of the pulse), and stayed monomeric until the appearance of the dimers (results not shown). This rules out the possibility that calnexin and calreticulin bind different Tg molecules present in transient Tg aggregates.

Although these data do not rule out the possibility that the two lectin-type chaperones are associated with one other and bind as a complex to Tg, they do show that calnexin and calreticulin co-exist in the same complex with newly synthesized Tg.

Figure 7 Newly synthesized Tg associates not only with calnexin and calreticulin, but also with BiP and GRP94

FRTL-5 cells routinely grown as outlined in the Experimental section were either cross-linked with DSP or mock-treated, lysed and immunoprecipitated (IP) with anti-Tg antibodies covalently coupled to CNBr–Sephacryl. The immunoprecipitates were resolved by SDS/PAGE and subjected to Western blotting (WB) using anti-Tg, anti-GRP94, anti-calnexin or anti-BiP antibodies. The positions of Tg, GRP94, calnexin (CNX), BiP and molecular-mass markers (kDa) are indicated.

Thapsigargin perturbs the folding of newly synthesized Tg and its interactions with calnexin and calreticulin

We have demonstrated that treatment of FRTL-5 cells with thapsigargin depletes ER Ca²⁺ stores and inhibits the secretion of Tg by preventing its exit from the ER [26,27]. These results suggest that depleting ER lumen of Ca²⁺ may alter the folding of Tg. Indeed, thapsigargin triggers the unfolded protein response,

verified by the activation of JNK (c-Jun N-terminal kinase)/SAPK (stress-activated protein kinase) and nuclear factor- κ B [27], and by the up-regulation of BiP mRNA (results not shown).

We therefore examined the interactions of Tg with calnexin/calreticulin under conditions of Ca²⁺ depletion. FRTL-5 cells were pulse-labelled and chased in a nominally Ca²⁺-free medium in the absence or presence of thapsigargin, and cell lysates were immunoprecipitated with anti-calnexin and anti-calreticulin antibodies and resolved by SDS/PAGE. As shown in Figure 6, under control conditions (in a Ca²⁺-free medium), the kinetics of dissociation of Tg from calnexin and calreticulin (which reflect the time spent by Tg in the calnexin/calreticulin cycle) were very similar to those obtained in regular medium. However, in the presence of thapsigargin, whereas the amounts of immunoprecipitated calnexin and calreticulin were unaffected, the kinetics of dissociation of Tg from calnexin and calreticulin were accelerated. Premature exit of Tg from the calnexin/calreticulin cycle may have contributed to the misfolding of Tg.

To gain some insight into the mechanism responsible for the retention of Tg in the ER of thapsigargin-treated cells, we analysed the association of Tg with BiP and GRP94, which have been shown to mediate the ER retention of misfolded proteins in several systems. As a first step, we detected the association of Tg with BiP and GRP94 in FRTL-5 cells under normal conditions by Western blot analysis of anti-Tg immunoprecipitates. Anti-Tg immunoprecipitates probed with anti-BiP antibodies showed the presence of BiP (Figure 7, lane 6). Moreover, after chemical cross-linking with DSP, anti-Tg immunoprecipitates probed with anti-GRP94 antibodies showed the presence of GRP94 (Figure

Figure 8 Thapsigargin causes stabilization of Tg–BiP and Tg–GRP94 interactions

(A) FRTL-5 cells were labelled as outlined in the Experimental section and chased in nominally Ca²⁺-free medium in the presence or in the absence of 5 μ M thapsigargin (THAPS) for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-BiP antibodies (Ab). Immunoprecipitated proteins were resolved by SDS/6%–PAGE. The positions of Tg, BiP and molecular-mass markers (kDa) are indicated. (B) FRTL-5 cells were labelled and then chased in a nominally Ca²⁺-free medium in the presence or absence of 5 μ M thapsigargin for the indicated times. Intact cells were cross-linked with DSP and lysed. Cell lysates were subjected to immunoprecipitation with anti-GRP94 antibodies. Immunoprecipitates were resolved by SDS/7.5%–PAGE. The positions of Tg, GRP94 and molecular-mass markers (kDa) are indicated. (C) FRTL-5 cells were labelled and then chased in nominally Ca²⁺-free medium for the indicated times. Cells were lysed and Tg was immunoprecipitated. The immunoprecipitates were divided in two, digested with endoH or mock-digested, and resolved by SDS/5%–PAGE. The appearance of a slow-migrating endoH-resistant Tg band is shown.

7, lanes 3 and 4). As a control, in the same experiment the presence of calnexin in anti-Tg immunoprecipitates was assessed (Figure 7, lane 5). Therefore, under normal conditions, Tg associates with BiP and GRP94 in FRTL-5 cells.

Next, FRTL-5 cells were pulse-labelled and chased in a nominally Ca^{2+} -free medium in the absence or presence of thapsigargin. Cell lysates were immunoprecipitated with anti-BiP or anti-GRP94 antibodies, and we looked for co-precipitation of Tg by these antibodies. On SDS/PAGE, a labelled BiP band at approx. 78 kDa or a labelled GRP94 band at approx. 94 kDa could be observed (Figures 8A and 8B). A labelled band corresponding to newly synthesized Tg was present at 330 kDa (Figures 8A and 8B). In these co-immunoprecipitations, association of Tg with BiP and GRP94 was maximal at 10 min of chase, and declined thereafter. Notably, at 60 min of chase in the absence of thapsigargin, when Tg had already largely dissociated from GRP94 (Figure 8B, lane 4 compared with lane 2), the great majority of newly synthesized Tg was nevertheless still in the ER, as measured by sensitivity to endoH (Figure 8C, lane 4). These data (Figures 8B and 8C) establish that use of the cross-linker DSP does not non-specifically create associations between these two proteins (Tg and GRP94) simply by virtue of their being present in the same (ER) compartment, but rather there is a specific interaction that is time-dependent (indeed, association of Tg with BiP was detected even in the absence of cross-linker). However, under control conditions, while BiP and GRP94 dissociated from Tg with a half-time that reflected the time required for normal folding, in the presence of thapsigargin the BiP-Tg and GRP94-Tg interactions were extended over the entire time period explored.

DISCUSSION

In the present study we have demonstrated, using two differentiated thyroid cell lines, that Tg interacts with both calnexin and calreticulin. This is the first time that an endogenous glycoprotein has been found in ternary complexes with these chaperones in the ER of live cells. We also show that the high concentration of Ca^{2+} in the lumen of the ER is required for normal interaction of Tg with calnexin/calreticulin, since loss of ER Ca^{2+} causes premature exit of Tg from the calnexin/calreticulin cycle. On the other hand, loss of ER Ca^{2+} prolongs the association of Tg with BiP and GRP94. We suggest that this prolonged association with BiP and GRP94, probably in combination with prolonged binding of Tg to other chaperones, retains Tg in the ER. These results could explain the well-known effects of Ca^{2+} depletion on protein exit from the ER [32].

In co-immunoprecipitation experiments, the majority of newly synthesized Tg interacted with calnexin and calreticulin, with a quantitative preference for calreticulin. The finding that binding increased during the first 10 min of chase was not surprising, since a similar result has been reported for other glycoproteins interacting with calnexin and calreticulin, i.e. VSV-G protein [3], α_1 -antitrypsin, complement factor 3, transferrin [1] and HIV gp 160 [19]. The half-times of dissociation of Tg from calnexin and calreticulin fit well with the slow folding and subsequent exit from the ER of Tg measured by other criteria, such as resistance to endo H (Figure 4B). Moreover, other glycoproteins show half-times of dissociation from calnexin and calreticulin similar to that of Tg: transferrin dissociates from calnexin with a $t_{1/2}$ of 35 min, and HIV gp 160 dissociates from calnexin with a $t_{1/2}$ of 35 min and from calreticulin with a $t_{1/2}$ of 25 min [1,19].

By comparing the electrophoretic mobility of Tg under non-reducing and reducing conditions during a chase, we have shown that Tg folding involves the progressive rearrangement/

formation of disulphide bonds. This process occurs during dissociation of Tg from calnexin and calreticulin. A comparison of the kinetics of Tg dissociation from calnexin and calreticulin, and of Tg folding and dimerization, indicates that conformational maturation of Tg precedes oligomerization (Figure 4B). The bulk of Tg dimerization occurs only after a major proportion of Tg has dissociated from calnexin and calreticulin, and after the appearance of fully oxidized Tg. Thus calnexin and calreticulin appear to be directly involved in monomer folding, rather than in dimerization, of Tg. Calnexin and calreticulin have been found to contribute to the folding and oligomerization of proteins, with their action largely inferred from comparison of the kinetics of dissociation from chaperones and of oligomerization. Thus calnexin facilitates monomer folding of the subunit of the acetylcholine receptor, since its dissociation largely precedes assembly [33], while in the case of oligomerization of MHC class II complexes, calnexin remains associated with MHC class II molecules throughout the assembly process [34]. Similarly, calreticulin facilitates either the folding of HA early intermediates [17] or the final assembly of the antigen peptide on the class I loading complex [35].

The ER of most eukaryotic species contains both calnexin and calreticulin, but the reason for this is not entirely clear. One possibility is that they have a redundant action, since calnexin and calreticulin often bind to the same glycoprotein substrates [6–10,15]. However, there are a growing number of glycoproteins that bind to one or the other chaperone. VSV-G protein binds to calnexin, but not to calreticulin [2,6], and for some substrates a sequential mode of action of calnexin and calreticulin has been demonstrated [5,7,17,18]. The differences in binding to calnexin compared with calreticulin could be due either to presentation of different stretches of contextual amino acids or to oligosaccharides with different spatial orientations that favour the binding of membrane-constrained calnexin or soluble calreticulin [6,12,16].

However, proteins above a certain molecular size may have binding sites for calnexin and calreticulin that are sufficiently spatially segregated to allow simultaneous binding of both chaperones, thereby assisting the folding of different regions of the protein at the same time. Our data show that Tg, an endogenous glycoprotein with 22 potential glycosylation sites distributed along the length of the 330 kDa polypeptide (of which a majority are actually glycosylated [20]), forms ternary complexes with calnexin/calreticulin in the ER of live cells. Our experiments do not clarify the modality of formation of such ternary complexes, i.e. whether calnexin and calreticulin bind Tg as calnexin-calreticulin binary complexes, or whether each binds independently of the other. However, once the ternary Tg-calnexin-calreticulin complexes have formed, it is likely that both chaperones participate in the conformational maturation of Tg, given the multiplicity of calnexin/calreticulin binding sites on the Tg polypeptide. The finding that the kinetics of dissociation of Tg from calnexin and calreticulin were similar but not identical is consistent with the possibility that different domains of Tg may be differentially involved in binding to calnexin and calreticulin.

That Tg possess several independent folding domains is suggested by several findings. The Tg polypeptide contains three regions: the N-terminal 1196 residues containing ten repeats of approx. 60 amino acids with six cysteines in each in invariable positions (type 1 repeats); a mid-portion, containing two other types of cysteine-rich repeat (type 2 and 3 repeats); and a C-terminal region with considerable identity with acetylcholinesterase. The Tg type 1 repeat is very similar to a six-cysteine motif found in several proteins [36,37]. The disulphide-bonding pattern of this motif has recently been determined, and the

disulphide bonds are Cys1–Cys2, Cys3–Cys4 and Cys5–Cys6 [36,37]. Disulphide bonds in Tg have not been determined experimentally, but they are expected to be the same [37]. Other cysteine-rich domains present in a variety of proteins, known as EGF (epidermal growth factor)-like motifs, have a different disulphide bond pattern, i.e. Cys1–Cys3, Cys2–Cys4 and Cys5–Cys6 [38]. Therefore, in both cases, individual cysteine-rich repeats have internal disulphide bonds, suggesting that they fold as independent domains. The C-terminal part of Tg shares 31 % identity with acetylcholinesterase. Strikingly, all cysteine residues that participate in disulphide bonding in acetylcholinesterase are conserved in both proteins, suggesting a similar folding pattern [39]. Therefore these data suggest that individual cysteine repeats in the N-terminal portion and in the acetylcholinesterase-like domain of Tg form independent folding domains. Distinct domains are also present in the mature protein, perhaps as a result of their independent folding. The formation of the thyroid hormones 3,3',5-tri-iodothyronine and thyroxine implies the transfer of an iodophenyl group from a 3-moniodotyrosine or 3,5-di-iodotyrosine donor to a 3,5-di-iodotyrosine acceptor. This reaction is strictly dependent on the three-dimensional structure of Tg, and the conformation of a 'hormonogenic domain' must be preserved. To date, four acceptor sites have been identified: Tyr-5, Tyr-1291, Tyr-2553 and Tyr-2746. The donor sites have also begun to be identified, and it appears that they lie in proximity to the acceptor site, always in the context of the same major domains discussed above [40–42]. These data and those presented herein suggest the presence of autonomous folding domains in the Tg polypeptide, the folding of which is assisted by calnexin and calreticulin, acting together and at the same time in ternary Tg–calnexin–calreticulin complexes.

Our results suggest that Tg folds in the calnexin/calreticulin pathway, with the formation of ternary complexes. This mechanism may be common to several multi-domain glycoproteins.

Loss of Ca²⁺ from the ER elicited by thapsigargin (a specific inhibitor of ER Ca²⁺-ATPases) profoundly altered the chaperone-binding pattern of Tg, facilitating its exit from the calnexin/calreticulin cycle and stabilizing its interactions with BiP and GRP94. It is possible that the stabilized interactions with BiP and GRP94, and perhaps with other chaperones, mediate the retention of Tg in the ER. In this respect, it has been demonstrated previously that, by increasing the binding of Tg to either BiP or to GRP94 by selectively overexpressing either of these chaperones, the residence time of Tg in the ER is increased [24,43]. On the other hand, thapsigargin inhibits the interactions of Tg with calnexin/calreticulin. This finding is in line with various data in the literature. Ca²⁺ modulates the conformation of the luminal domains of calnexin [44] and calreticulin [45]. Ca²⁺ is required for the binding of calnexin to glycoproteins [46], and for the binding of calreticulin to laminin [47]. It is also needed for the binding of oligosaccharide substrates to calnexin and calreticulin [14]. However, Ca²⁺ may also be required for glycoprotein re-binding to calnexin/calreticulin. UDP-glucose:glycoprotein glucosyltransferase, which catalyses the reglucosylation of glycans on unfolded proteins, allowing their re-binding to calnexin/calreticulin, requires Ca²⁺ for activity [48]. All of these studies have been performed *in vitro*. *In vivo*, A23187, a Ca²⁺ ionophore, has been found to prevent the formation of complexes between calnexin and H-2L^d (L^d heavy chains of the H-2L locus) [49]. Therefore our data extend *in vivo* findings, showing an effect of Ca²⁺ on the interactions between calreticulin and glycoproteins. Our hypothesis is that folding in the calnexin/calreticulin pathway facilitates the maturation and export of Tg. In support of this hypothesis is the finding that Tg export is delayed in cells treated with CST [25]. Moreover, thapsigargin

treatment also inhibits the exit of Tg from the ER [26], and we find that such treatment causes the premature exit of newly synthesized Tg from the calnexin/calreticulin cycle and an impairment of Tg dimerization [26], consistent with a Tg folding defect.

Accumulation of cargo proteins in the ER occurs in a variety of diseases, such as primary congenital hypothyroidism, cystic fibrosis and familial hypercholesterolaemia. In about 20 % of cases of primary congenital hypothyroidism, a mutation is present in one of the genes encoding proteins essential in the biosynthesis of thyroid hormones. One of these proteins is Tg. Among the known Tg mutations, missense mutations in the mouse [50] and human [51] cause impaired intracellular transport of Tg. In these cases, it is likely that the mutation alters the folding of Tg, and that the misfolded protein is not competent to leave the ER. Understanding the pathogenesis of these diseases at the molecular level will involve resolution of the conformation of wild-type Tg and of the altered conformation of the mutated protein. However, the ordered interactions of Tg with molecular chaperones described here could be exploited to give a molecular description of the alterations of the folding pathway that occur in these diseases.

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