Multiple Molecular Chaperones Complex with Misfolded Large Oligomeric Glycoproteins in the Endoplasmic Reticulum*

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Thyroglobulin (Tg), the major protein secreted by thyroid epithelial cells and precursor of thyroid hormones, is a large dimeric glycoprotein with multiple disulfide bonds. The folding and assembly of this complex molecule begins in the endoplasmic reticulum (ER) and is likely to involve a variety of reactions catalyzed by molecular chaperones (Kuznetsov, G., Chen, L. B., and Nigam, S. K. (1994) J. Biol. Chem. 269, 22990–22995). By communoprecipitation in rat thyroid cells, we were able to demonstrate that BiP, grp94, ERp72, and grp170, four proteins believed to function as specific molecular chaperones, complex with Tg during its maturation. The same complex of the four putative chaperones with Tg was observed in cells treated with tunicamycin, indicating that these four ER chaperones stably associate with Tg when it is misfolded/miss-assembled due to inhibition of its glycosylation. BiP, grp94, and ERp72 were also found to associate with Tg in cells in which misfolding was induced by perturbing ER calcium stores. To determine if the assembly of a complex between the four chaperones and Tg under conditions of misglycosylation was unique to the maturation of this particular secretory protein or a more general phenomenon, adenovirus-transformed rat thyroid cells that do not synthesize Tg were analyzed. In these transformed cells, the only protein these same four chaperones were found to complex with was a protein of approximately 200 kDa. This protein was subsequently identified as thrombospondin, which, like Tg, is a large oligomeric secreted glycoprotein with multiple disulfide bonds. We therefore propose that these ER chaperones complex together with a variety of large oligomeric secretory glycoproteins as they fold and assemble in the ER.

The ER1 contains a number of lumenal proteins, many of which have been proposed to function as molecular chaperones in the folding and/or assembly of membrane and secretory proteins (1–6). Molecular chaperones are postulated to bind transiently to newly synthesized proteins, interact with the polypeptides as they fold through sequential cycles of binding and release, and finally release the proteins upon their folding and/or assembly (7). Some of these ER chaperones are thought to bind to exposed hydrophobic regions of proteins (grp78/BiP) (8, 9), whereas others are thought to participate in disulfide isomerization (protein disulfide isomerase, ERp72) (10, 11) and peptidyl-prolyl isomerization (FKBP12) (12, 13). Many of the ER chaperones have been shown to associate with secretory proteins transiting the ER (examples include BiP, grp94, ERp72, and calnexin) (6, 14, 16), but it has been unclear whether they work together as part of a large complex or separately. One difficulty has been that it has often been necessary to resort to cross-linking approaches to demonstrate associations of multiple ER chaperones with secretory proteins (6, 16, 17). Whether stable associations of multiple chaperones with secretory proteins occur during their folding and assembly in the ER is a question of considerable interest. Furthermore, it is unknown whether particular sets of chaperones work together on certain classes of proteins or whether the case for each protein is different.

In a previous study, we reported that several unrelated unfolded protein substrates bind to the same set of resident ER proteins on affinity columns (5). These ER proteins, which all exhibited characteristics of molecular chaperones (such as binding to denatured substrates and elution from them in an ATP-dependent manner), included BiP, grp94, ERp72, protein disulfide isomerase, and calreticulin. One of the substrates used in the study was thyroglobulin (Tg), a protein precursor of thyroid hormones produced by thyroid epithelial cells. In support of these in vitro findings, we subsequently demonstrated by cross-linking and immunoprecipitation that BiP, grp94, and ERp72 also associate with Tg in intact thyroid cells (6). Similar associations between Tg and these chaperones were observed in a cell culture model for epithelial ischemia when ATP levels declined to less than 10% of control (18). Nevertheless, it remained unclear whether these associations exist in the absence of cross-linking or severe ATP depletion.

Coordinate binding to substrates and coelution of a set of ER molecular chaperones (5), as well as their coordinate induction under conditions that perturb the internal environment of the ER (13), prompted us to hypothesize the existence of large macromolecular chaperone-containing complexes that are involved in maturation of certain classes of secretory and membrane proteins. The association of multiple resident ER proteins with Tg by cross-linking is consistent with this hypothesis. In the present study we investigated the involvement of multiple ER molecular chaperones in the maturation of large oligomeric secreted glycoproteins under conditions of misfolding in the ER. Thyroglobulin (19) typifies this class of

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1 The abbreviations used are: ER, endoplasmic reticulum; BiP, immunoglobulin-binding protein; grp, glucose-regulated protein; ERp, endoplasmic reticulum protein; FKBP, FK506-binding protein; DSP, di-thiobis(succinimidyl propionate); PBS, phosphate-buffered saline; DTt, dithiorthreitol; Tg, thyroglobulin; Tn, tunicamycin; TSP, thrombospondin; PAGE, polyacrylamide gel electrophoresis.
proteins, made by many specialized secretory cells. Like immunoglobulin chains (20, 21) and thrombospondin (22), it is a large oligomeric glycoprotein with multiple disulfide bonds. The size (Tg is ~660-kDa homodimer consisting of two subunits of ~330 kDa) and apparent complexity (extensive glycosylation, disulfide bonding, and other posttranslational modifications) of Tg suggest that its folding and assembly may involve many reactions catalyzed by multiple ER chaperones. Indeed, it appears as though Tg does, at least transiently, associate with BiP, grp94, ERP72, and calnexin during its maturation (6, 23); however, with the exception of BiP (6, 23, 24), stable associations with Tg have not been observed. Since such associations seemed more likely to occur when the ER transit time of Tg was prolonged, we examined conditions that perturb the internal environment of the ER and lead to accumulation of immature proteins in the ER lumen. Here, we report that following treatment of cells with tunicamycin, an agent that inhibits N-linked glycosylation in the ER, the same set of four ER molecular chaperones (BiP, grp94, ERP72, and grp170) stably complexes with two secreted proteins that exhibit strikingly similar structural characteristics. We therefore propose that this set of four ER chaperones is involved in a dynamic “folding complex” for many large oligomeric glycoproteins.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antiserum against BiP used for immunoprecipitation was purchased from Affinity Bioreagents (Neshanic Station, NJ). The mouse monoclonal antibody against BiP used for Western immunoblot analysis and rat monoclonal antibody against grp94 were obtained from Stress-Gen (Victoria, British Columbia). Rabbit polyclonal antiserum against grp170 was a gift from Dr. J. Subjeck (Roswell Park Cancer Institute). Rabbit polyclonal antiserum against ERP72 was kindly provided by Dr. M. Green (St. Louis University). Rabbit polyclonal anti-thrombospondin antiserum used for immunoprecipitation was a gift from Dr. J. Lawler (25) (Brigham and Women’s Hospital, Harvard Medical School), and the rabbit polyclonal anti-thrombospondin antiserum used for Western immunoblot analysis was kindly provided by Dr. V. M. Dixit (University of Michigan Medical Center). Rabbit polyclonal antiserum against thyroglobulin was purchased from DAKO Corp. (Carpinteria, CA). Secondary antibodies were obtained from Amersham Corp. The following materials were purchased from Sigma: thyroid-stimulating hormone, insulin, hydrocortisone, transferrin, somatostatin, glycyl-L-histidyl-L-lysine acetate, leupeptin, pepstatin A, antipain, and tunicamycin. DSP was obtained from Pierce. A23187 and thapsigargin were from Calbiochem.

EXPERIMENTAL PROCEDURES

Cell Culture and Metabolic Labeling—Three rat thyroid epithelial cell lines were used in this study: FRTL-5 and PCCS (both Tg-producing) and PCE1A (lacking the ability to synthesize Tg). FRTL-5 cell line was obtained from ATCC (Rockville, MD). PCCS and PCE1A thyroid cells were kindly provided by Dr. M. T. Berlinger (Università degli studi di Napoli, Italy). Thyroglobulin-producing cells, FRTL-5 (26) and PCCS (27), were maintained in Coon’s modified Ham’s F12 medium (Sigma) supplemented with 5% bovine calf serum (HyClone Laboratories, Logan, UT) and a six-hormone mixture consisting of thyrotropin (1 mU/ml), insulin (10 μg/ml), hydrocortisone (10 nm), transferrin (5 μg/ml), somatostatin (10 ng/ml), and glycyl-L-histidyl-L-lysine acetate (10 ng/ml) (28). PCE1A cells (lacking thyroglobulin) were grown in the same culture medium but without the six-hormone supplement (27). Cells were metabolically labeled with either 18–20 h in their respective growth media or for 30 min in the serum-, methionine-, and cysteine-free medium in the presence of [35S]methionine/cysteine (200 μCi/ml, Expre35S-35S labeling mix, DuPont NEN).

Cross-linking and Immunoprecipitation—Cell monolayers were washed twice with the phosphate-buffered saline (PBS) and suspended in 1 ml of PBS, pH 7.4, containing 5 mM EDTA and 5 mM EGTA (6). The cells were incubated with the cross-linking agent, DTT (100 μg/ml), or vehicle (dimethyl sulfoxide, final concentration of 1%) for 30 min at room temperature. The cross-linking reaction was stopped by incubation with 100 mM Tris/HC1, pH 7.5, for 15 min. Cells were then lysed on ice by addition of 1% Triton X-100 in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 5 μg/ml each leupeptin, pepstatin A, and antipain). Prior to immunoprecipitation, samples were incubated with protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) to remove any material that binds nonspecifically to protein A. Samples were incubated with various antisera for 1 h at 4°C followed by incubation with protein A-Sepharose for 1 h at 4°C. The pellets were washed in PBS, suspended in sample buffer (29) containing 50 mM dithiothreitol, heated to 100°C for 10 min and analyzed by SDS-PAGE (29). Protein-associated radioactivity was analyzed on a PhosphorImager using the Image-Quant software package (Molecular Dynamics). Images were processed using the Adobe Photoshop™ software and printed with the Fujix Pictography 3000 color printer.

RESULTS AND DISCUSSION

Under conditions which perturb the folding and secretion of Tg, existing interactions of this large dimeric secretory protein with ER molecular chaperones would be expected to be prolonged and more stable, and therefore easier to detect without the use of cross-linking reagents. Inhibitors of glycosylation and agents that perturb ER Ca2+ stores create such conditions, and we have employed both types of agents to study the involvement of ER chaperones in Tg maturation. The nucleoside antibiotic tunicamycin inhibits N-linked glycosylation in the ER by blocking the formation of the lipid-linked oligosaccharide donor through specific inhibition of the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to dolichyl phosphate (30). After tunicamycin treatment, misglycosylated proteins accumulate in the ER (31), which is accompanied by the increased synthesis of ER molecular chaperones (13, 36), including BiP, grp94, and ERP72. Thus, since mature Tg is extensively glycosylated, tunicamycin treatment would be expected to severely affect its conformation and stability and to facilitate detection of interactions of Tg with molecular chaperones of the ER without the use of cross-linking agents. Two rat thyroid epithelial cell lines that synthesize and secrete Tg were used in our study: FRTL-5 and PCCS. Our findings in both cell lines were virtually identical; therefore, below we present results obtained with only one of each type of cell.

A relatively low dose of tunicamycin (1 μg/ml) was chosen. A recently published study (24) reported that as little as 0.5 μg/ml tunicamycin results in a complete inhibition of N-linked glycosylation of Tg. At the same time, a number of studies in recent years have shown that concentrations of tunicamycin similar to the one we have chosen result in virtually complete
inhibition of N-linked glycosylation without a significant effect on overall protein synthesis (32, 33). When FRTL-5 cells were treated with 1 \mu g/ml tunicamycin, Tg was found intracellularly in a lower molecular weight (unglycosylated) form that could be clearly distinguished by SDS-PAGE from its glycosylated counterpart (Fig. 1A, lanes 10 and 12). Inhibition of N-linked glycosylation of Tg persisted for up to 5 h after the removal of tunicamycin from the culture medium (Fig. 1A, lane 11). Secretion of unglycosylated Tg was almost completely (>95%) blocked in tunicamycin-treated cells (Fig. 1A, lanes 3, 6, and 9) as well as in cells pretreated with tunicamycin for 16 h and then transferred to tunicamycin-free medium for up to 5 h (Fig. 1A, lanes 2, 5, and 8). Thus, under the conditions used in our study, tunicamycin induces complete inhibition of N-linked glycosylation of Tg and completely blocks its secretion, and these two effects are not reversed during the time periods employed in the experiments described below. By immunofluorescence, the unglycosylated Tg localized largely to the ER (Fig. 1B). Furthermore, both Tg and BiP appeared to associate into larger macromolecular complexes when analyzed by sucrose density gradient centrifugation (Fig. 1C). In control cells, Tg sedimented largely in fractions 8–12 with a small amount of the protein present in fractions 13–15 and in fraction P. Fraction P represents material pelleted at the bottom of the gradient as a result of transient association of Tg into large complexes in the course of its normal maturation (34). The majority of intracellular BiP in untreated cells was found in fractions 3–8, with a small amount present in heavier fractions (fractions 9–12 and the pellet fraction, P). Following treatment of cells with tunicamycin, both Tg and BiP have shifted to heavier fractions of the gradient, and there was a marked increase in the amounts of both proteins found in the pellet fraction of the gradient. Together, these data suggested that tunicamycin treatment led to formation of large macromolecular complexes containing unglycosylated Tg and possibly ER molecular chaperones that were retained in the ER.

To ensure that the formation of Tg-containing macromolecular complexes in tunicamycin-treated cells does not represent nonspecific protein aggregation resulting from cell injury, we measured cell viability and overall cellular secretory activity in both treated and untreated cells. To assess cell viability, a trypan blue exclusion assay was performed following treatment of FRTL-5 cells with 1 \mu g/ml tunicamycin and chased for 4 h in the presence or absence of excess unlabeled methionine/cysteine and in the presence (lanes 2 and 3) or absence (lane 1) of tunicamycin (1 \mu g/ml). Aliquots of culture media were subjected to SDS-PAGE followed by autoradiography. Lane 3 is identical to lane 2 but overexposed to help in visualizing polypeptide bands. Arrows denote positions of Tg and the three additional polypeptide bands (designated p1, p2, and p3) secreted by thyroid cells. The amounts of radioactivity associated with Tg and bands p1, p2, and p3 were quantified using Molecular Dynamics PhosphorImager and the Image Quant software package and are presented in panels B and C. B, altered secretory profile of tunicamycin-treated cells. Open bars, control; closed bars, tunicamycin-treated. The relative amounts of four secreted proteins (Tg and bands 1, 2, and 3) are shown (expressed as percent of total in each lane). C, the absolute amounts of 35S-labeled bands corresponding to Tg, and bands p1, p2, and p3 in control (open bars) and tunicamycin-treated (closed bars) are shown.
Fig. 3. Associations of Tg with the molecular chaperones of the ER in stressed and unstressed thyroid cells. Uncross-linked (lanes 1, 3, 6, 8, 10, and 12) and cross-linked (lanes 2, 4, 5, 7, 9, and 11) lysates from [35S]methionine-labeled, untreated (lanes 1, 2, 3, 6, 7, 9, and 10) or tunicamycin-treated (lanes 3, 4, 7, 8, 11, and 12) FRTL-5 cells were subjected to immunoprecipitation with anti-Tg (lanes 1–4), anti-BiP (lanes 5–8), or anti-ERp72 (lanes 9–12) antisera. Note that lanes 5–12 containing cross-linked samples precede lanes containing uncross-linked samples. Arrows denote positions of Tg, p200, grp170, grp94, BiP, and ERp72; the asterisk denotes an unidentified polypeptide band of approximately 180 kDa that was immunoprecipitated with anti-ERp72 antisera but not with the anti-Tg or anti-BiP antisera.

Fig. 4. Identification of polypeptides coimmunoprecipitated with Tg and ER molecular chaperones. FRTL-5 cells were incubated with or without 1 μg/ml tunicamycin for 18 h. Cells were then treated with or without cross-linking reagent, lysed, and subjected to immunoprecipitation with anti-Tg (lanes 6–9), anti-BiP (lanes 10–13), or anti-ERp72 (lanes 14–17). Rat liver rough microsomes (lane 1), cell lysates (lanes 2–5), and immunoprecipitates (lanes 6–17) were subjected to 5% SDS-PAGE followed by Western immunoblotting with various antisera. A, anti-Tg; B, anti-grp170; C, anti-grp94; D, anti-BiP, E, anti-ERp72. Results of two separate experiments are combined in this figure.

Additional polypeptide bands were present on the SDS gel, although by far the Tg band (~300 kDa) was the most prominent. In control cells, the 300-kDa band represented approximately 75% of total labeled material secreted (Fig. 2B). Treatment of cells with tunicamycin reduced secretion of all polypeptides, but to various degrees (Fig. 2A, lanes 2 and 3). Secretion of three other secretory products of FRTL-5 cells (denoted by arrows designated p1, p2, and p3) was compared to that of Tg. In tunicamycin-treated cells, the amount of Tg present in culture medium was reduced by almost 200-fold (Fig. 2C). In contrast to Tg, secretion of polypeptides p1 (~112 kDa), p2 (~100 kDa), and p3 (~80 kDa) was reduced by only 1.7–2.5 fold (Fig. 2C). As shown in Fig. 2B, the secretory profile of tunicamycin-treated cells is significantly different from that of control preparations. Following treatment with tunicamycin, Tg becomes a minor secretory product, representing only ~5% of total secreted protein, while other polypeptides (like bands p1, p2, and p3) represent a substantial portion of total secretory material. Thus, different secretory proteins of thyroid cells exhibit vastly different sensitivities to tunicamycin treatment. These findings indicate that the inhibition of N-linked glycosylation by tunicamycin results in selective inhibition of secretion rather than in a nonspecific alteration of cellular function.

Together with the lack of toxicity of tunicamycin (as estimated by the trypan blue exclusion assay), these findings suggest that the action of tunicamycin on thyroid cells is specific and also support the validity of using tunicamycin as a tool to induce stable associations of Tg with the ER molecular chaperones.

The proteins associated with the unglycosylated Tg intermediate retained in the ER of tunicamycin-treated cells were then analyzed by coimmunoprecipitation with anti-Tg antibodies in the absence and presence of a thiol-cleavable chemical cross-linking agent (DSP). For initial identification of likely proteins associated with Tg in control and tunicamycin-treated cells, coimmunoprecipitation of Tg from metabolically labeled cells was performed. For subsequent positive identification of the coimmunoprecipitated polypeptides, Tg was immunoprecipitated from unlabeled cells, and the polypeptide bands were analyzed by Western immunoblotting. Fig. 3 and 4, respectively, show coimmunoprecipitations from metabolically labeled FRTL-5 cells and Western immunoblot analysis of the immunoprecipitates. In the absence of tunicamycin, the association of BiP, grp94, and ERp72 with Tg was seen after chemical cross-linking (Fig. 3, lanes 2, 3, and 4). This band was identified as the putative ER chaperone grp170 (Fig. 4B), a member of the glucose-regulated stress protein family that is thought to participate in folding and/or assembly of immunoglobulin chains (35). Thus, the large aggregates of unglycosylated Tg in the ER appeared to stably complex with four ER molecular chaperones. In Tg-containing cells, following tunicamycin treatment, BiP, grp94, and ERp72 could be seen even without cross-linkers (Fig. 3, lanes 3 and 4) and 12). This band was identified as the putative ER chaperone grp170 (Fig. 4B), a member of the glucose-regulated stress protein family that is thought to participate in folding and/or assembly of immunoglobulin chains (35). Thus, the large aggregates of unglycosylated Tg in the ER appeared to stably complex with four ER molecular chaperones.
though once again the associations were enhanced by cross-linking (Fig. 3, lane 7; Fig. 4, lane 13). Nevertheless, a stable complex of these four ER proteins with Tg appears to develop when normal Tg folding and/or assembly is perturbed by inhibition of its N-linked glycosylation. In addition, in BiP and ERp72 immunoprecipitates, a 200-kDa band was consistently observed. This band was subsequently identified (see below). The identity of an approximately 180-kDa polypeptide coimmunoprecipitated with ERp72 only has not yet been established (Fig. 3, lanes 9–12). It is possible that this 180-kDa polypeptide represents a secretory protein that specifically associates with ERp72 in the ER.

Some interactions between Tg and molecular chaperones, already increased after tunicamycin treatment, were further enhanced by cross-linking more than others. For example, treatment of tunicamycin-treated cells with cross-linker resulted in a marked further increase in amount of grp94 coimmunoprecipitated with Tg and BiP (Fig. 4C, lanes 8, 9, 12, 13) or in the amount of BiP coprecipitated with ERp72 (Fig. 4D, lanes 16, 17). The amount of grp170 co-precipitated with either Tg or BiP was unaffected by cross-linking (Fig. 4B, lanes 8, 9, 12, 13). Thus, the presumed misfolding of the Tg caused by misglycosylation affects the stability of interactions of the protein with molecular chaperones to different degrees.

grp170 is a glycoprotein (35), and after tunicamycin treatment it was indeed detected intracellularly as a lower molecular weight (unglycosylated) form (Fig. 4B, lanes 4, 5 versus lanes 2, 3). We could not detect an association of grp170 with either Tg or BiP in unstressed cells in the absence of cross-linking (Fig. 4B, lanes 6 and 10). However, treatment of unstressed cells with the cross-linking agent caused a small amount of the glycosylated form of grp170 to coimmunoprecipitate with both Tg and BiP (Fig. 4B, lanes 7 and 11). After treatment with tunicamycin, a substantial amount of grp170 coimmunoprecipitated with Tg and BiP (Fig. 4B, lanes 8 and 12), and this was not further increased by cross-linking (Fig. 4B, lanes 9 and 13). However, by Western immunoblot analysis, no grp170 was detected in any of the ERp72 immunoprecipitates (Fig. 4B, lanes 14–17), although a trace amount of polypeptide of identical molecular weight to grp170 was present in ERp72 immunoprecipitates from 35S-labeled, tunicamycin-treated cells (Fig. 3, lanes 11 and 12). Since grp170 and ERp72 were present in both anti-Tg and anti-BiP immunoprecipitates from tunicamycin-treated cells, failure to confirm by Western immunoblotting an association between ERp72 and grp170 suggested by autoradiographic analysis was puzzling. It is possible, however, that the amounts of these two ER proteins associating with each other is extremely low, and Western blotting is less sensitive than autoradiography in detecting these associations.

A long-term treatment with tunicamycin (such as used in the above described experiments) has been shown to induce the mRNA levels for ER molecular chaperones (including BiP, grp94, and ERp72) in various experimental systems (13, 36) as well as in rat thyroid epithelial cells (18). It is currently believed that the accumulation of misfolded and/or misassembled secretory proteins in the lumen of the ER serves as the signal and/or the trigger for the induction (36–38). Stable long-term associations of the ER molecular chaperones with their misfolded/misassembled protein substrates may also be a determining factor in signaling the induction of mRNA levels for these chaperones. We have therefore analyzed the associations of Tg with ER chaperones following treatment with other agents that induce their mRNA levels (presumably through causing accumulation of misfolded secretory proteins in the ER lumen): a Ca2+ ionophore A23187 and an inhibitor of the ER Ca2+-ATPase thapsigargin (39, 40). Both agents result in depletion of Ca2+ from the ER (41, 42) and are not known to induce aggregation in the ER lumen; in fact, it has been argued that calcium depletion may have the opposite effect (43). Following treatment of FRTL-5 cells with A23187, thapsigargin, or tunicamycin for 16 h, Tg was immunoprecipitated from uncross-linked cell lysates. Immunoprecipitates were analyzed by SDS-PAGE followed by Western immunoblotting with antisera against Tg (panel A), grp94 (panel B), BiP (panel C), or ERp72 (panel D).

To determine if the assembly of a complex involving the four chaperones was specific to Tg or represented a more general phenomenon, a group of adenovirus-transformed rat thyroid cells, PCE1As (27), were studied. These cells have lost their ability to synthesize and secrete Tg and no longer depend on thyroid-stimulating hormone for growth following transfection with the virus (27). As expected for these cells, no Tg was detected by immunoprecipitation (Fig. 6, lanes 1–4) or Western immunoblotting (data not shown). However, in 35S-methionine-labeled immunoprecipitates, the same complex of BiP, grp94, ERp72, and grp170 was observed in association with other bands (Fig. 6, lanes 8 and 12). An unidentified 180-kDa band was only seen in ERp72 immunoprecipitates (Fig. 6, lanes 9–12), but the most consistently observed band seen in both BiP and ERp72 immunoprecipitates migrated at approximately 200 kDa (Fig. 6, lanes 5–12). This band had also been visualized in BiP and ERp72 immunoprecipitates of 35S-methionine-labeled FRTL-5 cells that secrete Tg (Fig. 3, lanes 9–12).
The latter seemed a more plausible hypothesis since we could not detect an association of p200 with Tg (data not shown). Like Tg, the 200-kDa protein only entered standard polyacrylamide resolving gels under reducing conditions, suggesting that it too was a highly disulfide-bonded protein that possibly oligomerized under non-reducing conditions (Fig. 7, lanes 1 versus 2).

One such protein made by thyroid and other cells is a member of the thrombospondin protein family, thrombospondin-1 (TSP-1), a trimeric, multiply disulfide-bonded glycoprotein (22, 44). Native TSP-1 is a homotrimeric protein of 450 kDa, and its monomeric size of approximately 190–200 kDa corresponds to the p200 polypeptide band present on reducing gels. By immunoprecipitation and Western blotting, the 200-kDa band was subsequently indeed identified as TSP-1 (Fig. 7, panels B and C).

To summarize, the existence in thyroid cells of complexes containing these four ER chaperones was not dependent on Tg. In searching for other proteins that might associate with the same four chaperones, it was very striking to us that the only major protein detected turned to have many characteristics similar to Tg, for Tg to TSP-1 is also a large oligomeric secreted glycoprotein. Thus, TSP-1 would be expected to require reactions very similar to Tg for proper folding and/or assembly in the ER, consistent with our finding that the same set of ER chaperones complexes with both secretory proteins. Based on these results, we propose that BiP, grp94, ERP72, and possibly grp170 participate in complexes involved in the maturation of these two secretory glycoproteins. On the basis of similarities between Tg and TSP we hypothesize that, in general, large oligomeric glycoproteins may follow the same pattern of interaction with these four molecular chaperones as they pass through the ER. Two types of interactions are possible: concurrent and sequential. In the case of thyroglobulin, one would expect a heterogeneous population present in the ER lumen at all times, each subpopulation consisting of a certain intermediate in post-translational processing of the protein. Each subpopulation would then associate with a chaperone or a subset of the four chaperones. In support of this hypothesis is the fact that immunoprecipitations with different antibodies yielded different sets of proteins. For example, while all four chaperones coprecipitated with Tg, grp170 did not coprecipitate with ERP72. ERP72 and grp170 might therefore bind different intermediates in Tg maturation that are present at different time points along the maturation pathway, while binding of Tg to BiP and to grp94 occupies a broader time period and overlaps with binding to both ERP72 and grp170. Sequential action of chaperones like BiP and grp94 has been suggested in maturation of immunoglobulin light chains (17), and sequential action of calnexin and BiP has been suggested in maturation of thyroglobulin (23). Our findings, and the notion that chaperones recognize distinct features within polypeptides, are also consistent with a model of interaction in which various chaperones or sets of chaperones successively complex with various intermediates in maturation of oligomeric glycoproteins.

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FIG. 6. Associations between ER molecular chaperones in thyroid cells lacking thyroglobulin. PCE1A cells were metabolically labeled in the presence or absence of 1 μg/ml tunicamycin for 16 h. Tg (lanes 1–4), BiP (lanes 5–8), and ERP72 (lanes 9–12) were immunoprecipitated from cross-linked and uncross-linked cell lysates and subjected to 5% SDS-PAGE. Protein-associated radioactivity was analyzed on PhosphorImager using the Image-Quant software package (Molecular Dynamics). Arrows denote positions of p200, grp170, grp94, BiP, and ERP72.

FIG. 7. Identification of p200 as TSP-1. A. BiP was immunoprecipitated from metabolically labeled, uncross-linked PCCl3 cell lysates. Immunoprecipitates were resuspended in sample buffer either containing 50 mM DTT (reducing conditions, lane 1) or lacking DTT (non-reducing conditions, lane 2). Reduced and non-reduced samples were resolved on 4% SDS-PAGE followed by autoradiography. B. TSP-1 was immunoprecipitated from metabolically labeled, uncross-linked PCCl3 or PCE1A cell lysates. Immunoprecipitates were resuspended in sample buffer either containing (lanes 3 and 4) or lacking (lanes 5 and 6) 50 mM DTT. Reduced and non-reduced samples were analyzed on 5% SDS-PAGE followed by autoradiography. C. BiP was immunoprecipitated from unlabeled PCCl3 or PCE1A cell lysates treated with or without cross-linking agent, DSP. Total PCCl3 or PCE1A cell lysates (lanes 7 and 8) and BiP immunoprecipitates from PCCl3 or PCE1A cells (lanes 9–12) were subjected to 5% SDS-PAGE under reducing conditions followed by Western immunoblotting with anti-TSP-1 antisera.
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