

Identification of ERp29, an Endoplasmic Reticulum Luminal Protein, as a New Member of the Thyroglobulin Folding Complex*

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Folding and post-translational modification of the thyroid hormone precursor, thyroglobulin (Tg), in the endoplasmic reticulum (ER) of the thyroid epithelial cells is facilitated by several molecular chaperones and folding enzymes, such as BiP, GRP94, calnexin, protein disulfide isomerase, ERp72, and others. They have been shown to associate simultaneously and/or sequentially with Tg in the course of its maturation, thus forming large heterocomplexes in the ER of thyrocytes. Here we present evidence that such complexes include a novel member, an ER-resident luminal protein, ERp29, which is present in all mammalian tissues with exceptionally high levels of expression in the secretory cells. ERp29 was induced upon treatment of FRTL-5 rat thyrocytes with the thyroid-stimulating hormone, which is essential for the maintenance of thyroid cells and Tg biosynthesis. Chemical cross-linking followed by the cell lysis and immunoprecipitation of ERp29 or Tg revealed association of these proteins and additionally, immunocomplexes that also included major ER chaperones, BiP and GRP94. Sucrose density gradient analysis indicated co-localization of ERp29 with Tg and BiP in the fractions containing large macromolecular complexes. This was supported by immunofluorescent microscopy showing co-localization of ERp29 with Tg in the putative transport vesicular structures. Affinity chromatography using Tg as an affinity ligand demonstrated that ERp29 might be selectively isolated from the FRTL-5 cell lysate or purified luminal fraction of rat liver microsomes along with the other ER chaperones. Preferential association with the urea-denatured Tg-Sepharose was indicative of either direct or circuitous ERp29/Tg interactions in a chaperone-like manner. Despite the presence of the C-terminal ER-retrieval signal, significant amounts of ERp29 were also recovered from the culture medium of stimulated thyrocytes, indicating ERp29 secretion. Based on these data, we suggest that the function of ERp29 in thyroid cells is connected with folding and/or secretion of Tg.

Proper folding, post-translational modifications, and oligomerization of the secretory proteins in the endoplasmic reticulum (ER)¹ are essential prerequisites for their recruitment into the transport vesicles heading toward the cell exterior (1). Quality control of potential cargo proteins is accomplished by the molecular chaperones that monitor fidelity of the protein folding and prevent premature export of incorrectly folded or incompletely assembled secretory proteins from the ER (2). Circumstantial evidence, such as inducibility in certain cell types under the ER stress conditions (3), high expression in the secretory tissues (4, 5), and co-localization with the ER chaperones (3), suggests that a recently discovered, ubiquitously expressed endoplasmic reticulum luminal protein, ERp29, may complement this group of ER chaperones.

ERp29 cDNA was originally cloned from the rat liver (3, 6) and enamel cells (7), and the *ERp29* gene was shown to be highly conserved in all studied mammalian species (3–5). For instance, rat ERp29 and its human ortholog, originally termed ERp28 (8), are 90% identical on the amino acid sequence level (9).

ERp29 consists of two domains of which the N-terminal domain of ERp29 resembles the thioredoxin module of protein disulfide isomerase (PDI), although without the active site double cysteines, indicating the lack of PDI-like redox function (9). The C-terminal domain represents a novel all-helical fold that is absent in human PDI but found in the P5-like PDIs and also in the *Drosophila* analog of ERp29, Windbeutel (10). The function of this domain remains unclear, although its C-terminal sequence, KEEL, which is a conserved variant of the ER retrieval signal, KDEL (11), most probably confers ER retention to ERp29.

Recently the *ERp29* gene has been shown to be activated upon the treatment of rat thyroid epithelial cells by the thyroid-stimulating hormone (TSH) (12). The latter is essential for the optimal growth of thyrocytes and expression of thyroid-specific genes including the gene encoding a thyroid prohormone thyroglobulin (Tg), a 660-kDa homodimeric secretory protein (13). The proper folding and assembly of the transport-competent Tg dimers is controlled by the major ER molecular chaperones and folding enzymes, such as BiP, GRP94, calnexin, ERp72, and PDI. Simultaneous and/or sequential interactions of Tg with such helper proteins lead to the transient formation of the large macromolecular heterocomplexes in the ER (14–16).

A high level of *ERp29* gene expression in thyrocytes (5) and its transcriptional regulation by TSH (12) suggested that

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¹ The abbreviations used are: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; Tg, thyroglobulin; TSH, thyroid-stimulating hormone; BFA, brefeldin A; DSP, dithiobis(succinimidyl) propionate; 6H, six-hormone; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

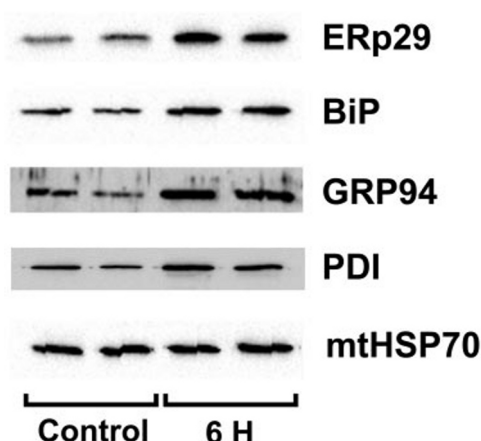


FIG. 1. **Induction of ERp29 and ER chaperones by hormones.** FRTL-5 cells were grown with or without the hormonal mixture (6H) to 70–80% confluency. The cells were subsequently lysed, electrophoresed, and immunoblotted with corresponding antibodies. Equal amounts of total protein were applied to each lane.

ERp29 may be implicated, along with other ER chaperones, in the maturation and/or secretion of Tg. Therefore we carried out immunoprecipitation of ERp29 and Tg, sucrose density gradient fractionation of the cross-linked thyrocytes, and affinity chromatography on the denatured Tg-Sepharose, which indicated involvement of ERp29 in the heterogeneous Tg folding complexes. Moreover, we found co-localization of ERp29 with Tg in the putative intracellular transport structures as evidenced by immunofluorescence microscopy and, additionally, secretion of considerable amounts of ERp29 from thyrocytes. These findings for the first time suggest a distinct function for ERp29 connected with the folding and/or export of secretory proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—FRTL-5 Fisher rat thyrocytes were cultivated in the Coon's modified Ham's F-12 medium containing 10% fetal bovine serum (Invitrogen) with the addition of the six-hormone (6H) mixture: thyrotropin (TSH) (1 milliunit/ml), transferrin (5 μ g/ml), somatostatin (10 ng/ml), glycyl-1-histidyl-1-lysine (10 ng/ml), hydrocortisone (10 nM), and insulin (10 μ g/ml). All of the hormones were purchased from Sigma.

Isolation of the Luminal Fraction of the Rat Liver Microsomes—The microsomes were isolated from the livers of male Sprague-Dawley rats as described in detail elsewhere (17). They were subsequently washed in 100 mM sodium pyrophosphate, pH 7.4, resuspended in 20 mM Tris-HCl, 1 mM dithiothreitol, 150 mM NaCl, pH 8.0, and sonicated. Solubilized microsomes were recentrifuged at 100,000 \times g, and the supernatant was designated as a luminal fraction.

Metabolic Labeling and Immunoprecipitation—The cells were grown with or without the 6H mixture 3 days prior to the experiment, washed with PBS, incubated for 1 h in the methionine-free medium, and labeled with 100 μ Ci/ml [³⁵S]methionine (Amersham Biosciences) for 4 h. The cells were washed twice with PBS and cross-linked with the 200 μ g/ml of the homobifunctional, thiol-cleavable, membrane-permeable agent, DSP (Pierce) in PBS at room temperature for 30 min. The reaction was stopped by the 100 mM Tris-HCl, pH 7.5, and the cells were lysed by the 1% Triton X-100 in PBS containing protease inhibitors mixture ("Complete," Roche Molecular Biochemicals) for 15 min, scraped, and centrifuged for 10 min at 17,000 \times g. The ensuing supernatant was used for the immunoprecipitations with the polyclonal anti-ERp29 IgGs conjugated with the BrCN-Sepharose (Amersham Biosciences) or polyclonal anti-human Tg (Dako) for 16 h. 25% protein A-Sepharose was added to the samples treated with anti-Tg and incubated for 1 h. The immunocomplexes were washed three times with the lysis buffer and once in PBS and water and eluted by the SDS-PAGE sample buffer with the subsequent reducing SDS-PAGE. The proteins were transferred to the nitrocellulose membrane and analyzed by the Fuji BAS-1800 phosphorimager.

Sucrose Density Gradient—Cross-linked or control FRTL-5 cell lysates including protease inhibitors were layered on top of the discontinuous 5–20% sucrose gradients prepared in PBS (six 1-ml layers with

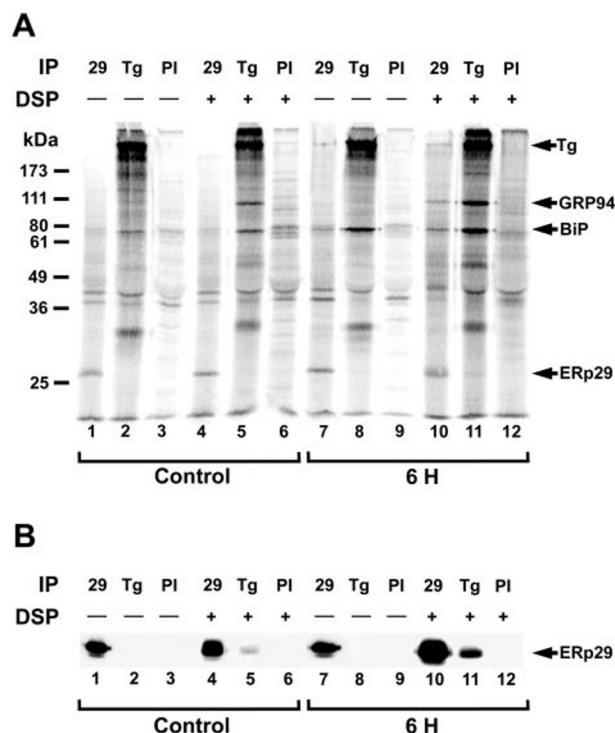


FIG. 2. **ERp29 association with Tg and chaperones.** FRTL-5 cells were treated as indicated in the legend to Fig. 1, labeled with [³⁵S]methionine for 4 h, and cross-linked with DSP, and the cell lysates representing equal amounts of total labeled proteins were immunoprecipitated with BrCN-immobilized ERp29 polyclonal IgGs, polyclonal anti-Tg (Tg), or preimmune rabbit serum (PI). The proteins were resolved by 8.5% reducing SDS-PAGE and transferred to the nitrocellulose membrane. The identities of co-immunoprecipitated BiP, GRP94, and Tg were confirmed by immunoblotting (results not shown). A, autoradiograph of the membrane. B, Western blot analysis of the same membrane using ERp29-specific antibodies.

the 3% sucrose increments) and centrifuged for 16 h in the Beckman SW40TI swing-out rotor at 100,000 \times g. The centrifuge tubes were punctuated in the bottom, and 12 \times 0.5-ml fractions were collected and precipitated by 10% (final concentration) trichloroacetic acid. The pellets were washed by ice-cold acetone, resuspended in the SDS-PAGE sample buffer, and analyzed by reducing SDS-PAGE followed by Western blot.

Affinity Chromatography—Affinity interactions with thyroglobulin and histone ligands were scrutinized as in Ref. 15 with minor modifications. FRTL-5 cell lysate ($\sim 2 \times 10^6$ cells) or luminal fraction (50 μ g of protein) of the rat liver microsomes was incubated for 16 h at 4 $^{\circ}$ C on the rotating wheel with 50 μ g (wet weight) of non-denatured or denatured (30 min in 6 M urea and 1 M β -mercaptoethanol) Tg- or histone-Sepharose beads (Sigma) in the binding buffer consisting of 20 mM Tris-HCl, 150 mM NaCl, 1 mM dithiothreitol, and protease inhibitors mixture, pH 8.0. The beads were subsequently washed with 2 ml of the same buffer and eluted with 0.5 ml of 1 mM ATP in the binding buffer including 2 mM MgCl₂ and 0.5 mM CaCl₂, followed by boiling for 1 min in the 50 μ l of SDS solubilization buffer. Flow-through, wash, and ATP eluate fractions were concentrated by acetone precipitation and dissolved in the SDS solubilization buffer. All of the fractions were analyzed by SDS-PAGE with subsequent immunoblotting.

Western Blot—The proteins were resolved by SDS-PAGE, transferred to the nitrocellulose membrane, and probed with antibodies against ERp29 (3), Tg, BiP (anti-peptide rabbit polyclonals, StressGen), GRP94 (rat monoclonals, StressGen), PDI (monoclonals, Affinity BioReagents), and mitochondrial HSP70 (polyclonals; Affinity BioReagents). Immunoreactive bands were visualized by the SuperSignal enhanced chemiluminescence according to the manufacturer's specifications (Pierce) using luminescent image analysis system LAS 1000+ (Fujifilm).

Immunofluorescent Microscopy—FRTL-5 cells were grown in the medium containing the 6H mixture on coverslips to 30–40% confluency, washed twice with PBS, and fixed for 10 min in 2% formaldehyde followed by a brief rehydration in PBS. The cells were permeabilized for

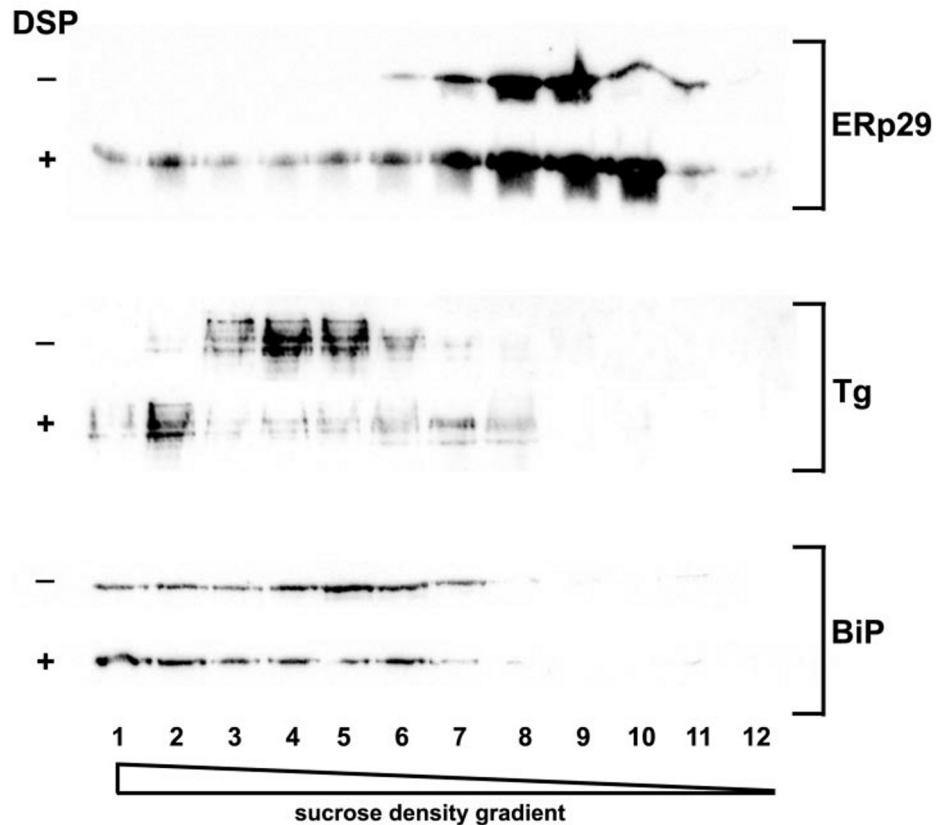


FIG. 3. Sucrose density gradient analysis of ERp29, BiP, and Tg in the cross-linked thyrocytes. FRTL-5 cells were grown in the 6H-containing medium to 70–80% confluency, cross-linked by DSP, lysed, layered on the top of the 5–20% sucrose gradient, and centrifuged to equilibrium. Twelve fractions were collected, and proteins were precipitated by trichloroacetic acid treatment and analyzed by 8.5% reducing SDS-PAGE followed by Western blot.

10 min in 0.2% Triton X-100, washed with TBS, and subsequently probed with primary antibodies in 2% bovine serum albumin, 0.02% Tween 20, 10% glycerol in PBS for 30 min. After five 2-min washes with PBS, the cells were incubated for 30 min with fluorochrome-conjugated secondary antibodies (Sigma) and washed again with PBS. DNA was stained by Hoechst 33258 (Sigma). All of the incubations were carried out at room temperature. For *in vivo* staining, the cells were fixed and incubated with primary and secondary antibodies in the humidified CO₂-incubator at 37 °C. The coverslips were mounted with VectraShield. Optical sectioning and three-dimensional reconstitution were carried out using a Zeiss Axiophot microscope. The images were captured with a PXL cooled CCD camera (Photometrix). Image processing was provided by the imaging program ST-FITC-Rhodamine-Hoechstbin1 (18), which produces both single and stereo-projected three-color images from a series of wide field pictures where the out-of-focus blur was removed by nearest neighbor deconvolution and the images were built up using a maximum intensity projection algorithm.

RESULTS

ERp29 Is Induced upon Stimulation of Thyrocytes—Stimulation of FRTL-5 cells by the mixture of six hormones including TSH revealed an approximately 2-fold increase of the immunodetectable ERp29 in accordance with the previously reported induction of mRNA synthesis under the same conditions (12) (Fig. 1). BiP, GRP94, and PDI levels where also elevated 2–3-fold as seen before (19, 20), whereas the amount of the mitochondrial HSP70 remained unchanged, indicating the specificity of the effect for the ER (Fig. 1). TSH alone was able to cause similar induction of ERp29 and ER chaperones in a dose-dependent manner, although the effect was less pronounced (results not shown).

ERp29 Associates with Tg, BiP, and GRP94—Concerted up-regulation of ERp29 and ER chaperones suggests a putative chaperone-related function for ERp29, in which case its association with the nascent Tg molecules might be expected. To investigate this possibility, we have immunoprecipitated Tg and ERp29 from the quiescent and 6H mixture-grown thyroid cells, which were metabolically labeled and chemically cross-linked prior to the lysis.

Anti-Tg co-precipitated BiP and GRP94 (the latter appeared in the Tg immunocomplexes only upon cross-linking) (16, 20). The amounts of these chaperones associated with Tg were further enhanced by hormonal stimulation (Fig. 2A, lanes 2, 5, 8, and 11). In addition, Tg immunocomplexes from the 6H mixture-grown and cross-linked cells display a weak labeled band migrating at the level of ~29 kDa. Immunoblotting experiments revealed its identity as ERp29 (Fig. 2B). Moreover, an ERp29-immunoreactive band was seen also in the cross-linked quiescent cells (Fig. 2B, lane 5). Thus, ERp29 appears to be a member of the Tg-containing heterocomplexes that also include BiP and GRP94. The complex formation was significantly enhanced by the hormone stimulation. The appearance of ERp29 band on the autoradiographs was relatively weak, either because of a weak association of ERp29 with Tg or because of the insufficient ³⁵S labeling of ERp29. However, Western blotting demonstrated rather strong immunoreactive staining.

The authenticity of the presented data was examined by immunoprecipitation experiments using ERp29 antibodies. Although the control cell lysates did not contain any co-precipitating Tg, a conspicuous Tg band appeared in the stimulated cells, which was additionally enhanced by DSP (Fig. 2A, lanes 7 and 10). Moreover, the immunocomplexes from the cross-linked samples precipitated by anti-ERp29 contained BiP and GRP94 similar to the Tg complexes (same lanes), and the appearance of GRP94 was dependent on the cross-linking as in the case of Tg immunoprecipitation. This indicates weak interactions of these proteins in the ER.

Because ATP is required for the dissociation of Tg from BiP (15) and some other chaperones, we immunoprecipitated Tg in the presence of 2 mM Mg/ATP or 50 units/ml apyrase/5 mM EDTA. The amount and content of Tg immunocomplexes remained unaltered (results not shown), indicating that ERp29 interaction(s) in the Tg multipartite complex is (are) ATP-independent. This is consistent with an earlier report where no

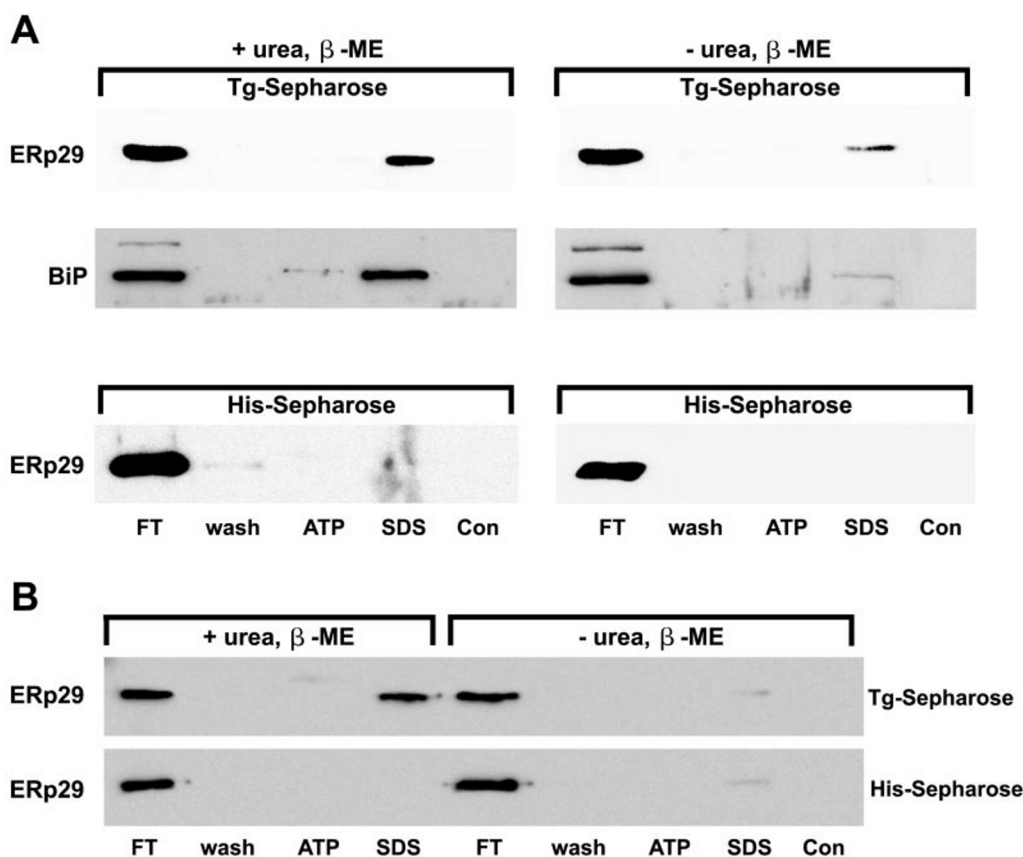


FIG. 4. Binding of ERp29 and BiP to the thyroglobulin and histone affinity resins. A, FRTL-5 cell lysate interacted with the denatured (with urea and β -mercaptoethanol (*ME*)) or native Tg or histone (*His*) immobilized on the Sepharose. Retained proteins were eluted with 1 mM ATP and SDS solubilization buffer subsequently. All of the fractions except SDS were precipitated with acetone, solubilized in the SDS solubilization buffer, and resolved by 12% SDS-PAGE followed by immunoblotting against ERp29 and BiP. *FT*, flow-through; *Con*, control binding of the cell lysate or microsomal lumen to the blocked BrCN-Sepharose demonstrated absence of the nonspecific binding of the examined proteins to the Sepharose matrix. B, luminal fraction of the rat liver microsomes underwent identical affinity binding procedure, and ERp29 was detected as described above.

ATP binding to ERp29 was found under *in vitro* conditions (8).

Co-immunoprecipitation of ERp29 with Tg and ER chaperones suggests the existence of the transient high molecular weight folding complexes in the ER. To examine this possibility, control and cross-linked FRTL-5 cells lysates were fractionated using sucrose density gradient centrifugation, and the resulting fractions were analyzed for the presence of ERp29, BiP, and Tg.

Tg was detected in the higher molecular weight fractions (Fig. 3, lanes 3–5) and was further shifted toward denser fraction (lane 2) upon cross-linking. Interestingly, a similar shift was observed also with ERp29 and BiP. In DSP-treated cells, a significant amount of ERp29 was distributed throughout the gradient with a conspicuous peak in the fraction 2, corresponding to the highest amount of Tg detected in the cross-linked sample. Relatively low levels of ERp29 in this fraction are consistent with a small amount of ERp29 co-immunoprecipitated with Tg. BiP was distributed more widely in the fractions obtained from the intact cells, which indicates more stable interactions in the complexes with different substrate proteins and/or ER chaperones. In line with these data, significant amounts of BiP were co-immunoprecipitated with Tg even from noncross-linked cells (Fig. 2). DSP treatment led to the further redistribution of BiP toward the heavier fractions with a peak detected in the fractions 1–2, also containing high amounts of Tg and ERp29.

ERp29 Interacts with Denatured Tg—To investigate further possible interactions of ERp29 within Tg complexes in the ER, we employed affinity chromatography using native or dena-

tured protein ligands such as thyroglobulin and histone coupled with a Sepharose matrix. It has been shown earlier that several ER chaperones and folding catalysts bind with high specificity to immobilized denatured Tg, histone, and other proteins (15). Many ER chaperones are ATP-binding proteins, and it was demonstrated that ATP hydrolysis is essential for the dissociation of BiP from its substrates (21). Thus, FRTL-5 cell lysate was allowed to bind to the denatured Tg-Sepharose in the batch adsorption manner, eluted first with 1 mM ATP and then with the SDS solubilization buffer. Ensuing fractions were tested for the presence of ERp29, BiP, and PDI.

In spite of the fact that the majority of ERp29 was found in the flow-through fraction, significant amounts were also detected in the SDS eluate, indicating its interaction with the affinity matrix (Fig. 4A). ATP was unable to dissociate ERp29 from the immobilized Tg. BiP was also found associated with the Tg-Sepharose and was eluted with ATP, although large amounts of protein remained attached to the affinity resin and were dissociated only by treatment with the SDS solubilization buffer (Fig. 4A). A similar elution profile was observed for PDI (results not shown). Interestingly, ERp29 was detected even in the eluates from the nondenatured Tg, although in lower amounts. Elution of BiP by ATP from the nondenatured matrix was below the detection level and barely detectable in the SDS eluate, which is in line with the demonstrated ability of BiP to interact exclusively with the misfolded substrates (21).

The binding of ERp29 to the Tg-Sepharose beads seems to be rather specific, because no interaction was observed with the nondenatured as well as denatured histone-Sepharose (Fig. 4A).

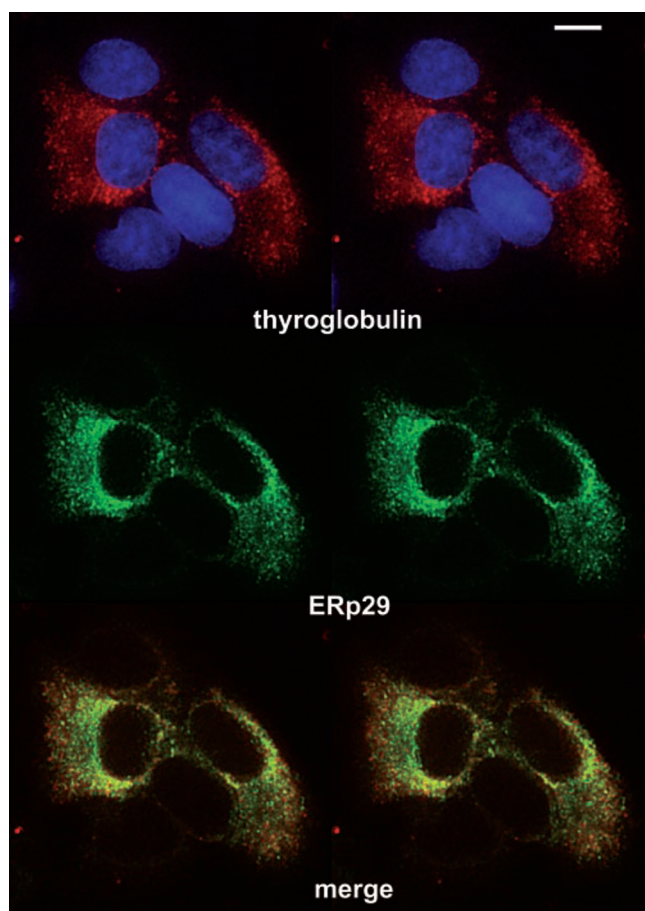


FIG. 5. **Co-localization of ERp29 and Tg.** FRTL-5 cells were fixed, permeabilized, and double-labeled with the polyclonal anti-ERp29, fluorescein isothiocyanate-conjugated anti-rabbit IgGs and monoclonal anti-Tg followed by TRITC-conjugated rabbit anti-mouse IgGs. Three-dimensional reconstruction of ERp29- and Tg-stained FRTL-5 cells as reconstituted from a series of nine mathematically deblurred optical sections $0.2 \mu\text{m}$ apart and projected as a pair of stereoisomages. The scale bar represents $10 \mu\text{m}$.

The latter matrix has been previously shown to bind several ER chaperones including BiP and PDI (15), as was also observed under our experimental conditions (results not shown).

We also examined interactions with the luminal fraction from the rat liver microsomes, which was free from the cellular components present in the total cell lysate. ERp29 was found associated with the denatured and to a lesser extent with the native Tg-Sepharose and eluted only with SDS solubilization buffer (Fig. 4B). Denatured histone-Sepharose was unable to retain ERp29 from the luminal fraction of rat liver microsomes (Fig. 4B).

ERp29 Co-localizes with Tg—Using immunofluorescence microscopy we have shown previously a typical ER distribution of ERp29 in the rat hepatoma cells (3). Immunofluorescent labeling of the permeabilized FRTL-5 cells with anti-ERp29 demonstrated a similar staining pattern (Fig. 5). However, in contrast to the hepatoma cells, where the staining was stronger in the perinuclear area, in the hormone-stimulated thyrocytes, additionally to the extensive staining of the perinuclear region, ERp29 also exhibited a punctate distribution throughout the cytoplasm. Double labeling of the same cells with anti-Tg showed an identical staining pattern, and merging of two fluorescent images resulted in the color shift characteristic for co-localizing proteins (Fig. 5). To establish the extent of the overlap, we carried out optical sectioning of the double-stained cells and reconstituted the three-dimensional distribution pat-

terns of ERp29 and Tg using stereo projection of the mathematically deblurred image stacks. This method is much more sensitive than the laser confocal imaging and allows high resolution comparison of the dense fluorescent signals. The images (Fig. 5) showed a very high degree of spatial co-localization of these proteins, apparently in the same vesicular transport structures.

ERp29 Is Secreted from the Thyrocytes—It has been previously reported that a number of ER-resident proteins (22–24) including PDI (Ref. 25 and references therein) are found in the culture media of the different primary and transformed cell lines. We examined media collected from the quiescent and hormone-stimulated cells for the presence of ERp29. Discernible amounts of the protein were found in the culture medium of the quiescent cells, which further increased upon the stimulation of cells with the hormonal mixture (Fig. 6A). The relative amount of ERp29 recovered from the 4-h medium was $\sim 11\%$ of the total (intracellular + secreted) ERp29. This corresponds to $\sim 3.5 \text{ pmol}/\sim 2 \times 10^6$ cells as quantified by densitometry of immunoreactive bands with the calibration curve based on the serial dilutions of the recombinant ERp29 (results not shown). Specificity of the ERp29 export was verified by testing media for the presence of other ER luminal proteins and mitochondrial HSP70. Among all tested proteins, only PDI was recovered from the medium, whereas BiP, GRP94, and mitochondrial HSP70 were not secreted (Fig. 6A). The kinetics of the ERp29 export from FRTL-5 cells is almost identical to the Tg and PDI secretion (Fig. 6, B and C). We examined medium from the cells treated with brefeldin A (BFA), the inhibitor of ER-to-Golgi transport to evaluate any contribution of cell leakage to the appearance of ERp29 in the medium. In 4 h BFA reduced the level of secretable ERp29, as well as Tg and PDI, down to 10–30% of the intact cell amounts (Fig. 6B). Additionally, analysis of the lactate dehydrogenase activity showed that only 1% of lactate dehydrogenase was released from the cells after 4 h, whereas ERp29 and PDI levels in the culture medium were estimated as 10–11% of the total amount.

DISCUSSION

A hypothetical involvement of ERp29 in the processing of the secretory proteins in the ER was hitherto supported by circumstantial evidence, such as co-localization with the ER chaperones and foldases, high expression in the secretory cells (4), and induction by ER stress (3). The data presented in the current study demonstrate for the first time that in the professional secretory cells, such as thyrocytes where the expression of ERp29 reaches its highest levels (5), ERp29 might be directly implicated in the process of maturation and secretion of the main export product of these cells, thyroid prohormone Tg.

The TSH-induced elevation of the ER chaperones in the thyroid cells is well documented and considered to be a part of the coordinated cell response to the increased synthesis of Tg (19, 20). It was suggested that the influx of nascent Tg molecules in the ER triggers the unfolded protein response machinery responsible for the transcriptional activation of the chaperone genes (14). We have observed a similar TSH-dependent up-regulation of ERp29 along with BiP, GRP94, and PDI, which is also consistent with the previously reported induction of the ERp29 transcript synthesis (12). Comparable accumulation of the unfolded proteins in the ER (ER stress) caused by the different agents inhibiting protein glycosylation or altering calcium homeostasis or redox environment was also found to stimulate ERp29 biosynthesis in other mammalian cells (3). It may therefore be speculated that up-regulation of ERp29 by TSH is coupled with the coordinated induction of the ER chaperones and PDI to maintain or even accelerate the folding rate of Tg.

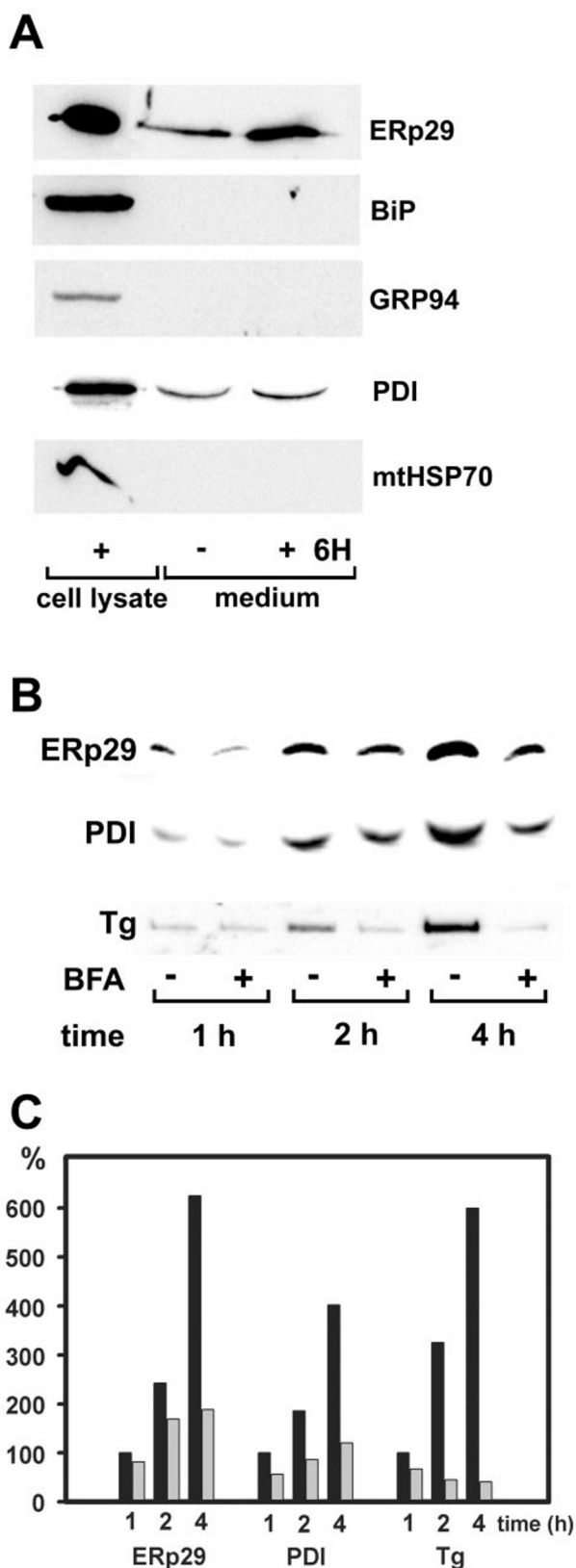


FIG. 6. ERp29 is secreted from the FRTL-5 cells. *A*, effect of the hormonal mix on the ERp29 and PDI secretion. FRTL-5 cells were treated by the hormones (6H) as indicated in the legend to Fig. 1, and 4 h after the change of media, the volumes representing equal amounts of cells were collected and centrifuged for 10 min at $17,000 \times g$, and supernatant was concentrated by acetone, resuspended in the SDS sample buffer, and immunoblotted with corresponding antibodies. The first lane from the left is a positive control representing cell lysates immunoblotted with the same antibodies. *B*, kinetics of ERp29, PDI, and Tg secretion. The cells were kept in the 6H-supplemented medium,

Folding of Tg is accomplished in the large macromolecular complexes containing simultaneously a number of molecular chaperones that control the folding state of Tg by repetitive binding and dissociation until the formation of the transport-competent, stable Tg dimers (14). Such transient interactions of molecular chaperones with Tg are usually weak (with the exception of BiP), and association with Tg is detected only if augmented by chemical cross-linking (16, 20). Stabilization of the noncovalent interactions by cross-linking was also essential for the detection of ERp29 in the Tg- and BiP-containing sucrose density fractions and in the Tg immunoprecipitates, suggesting the weak and transient nature of ERp29 associations. Transient binding of molecular chaperones to their substrates may be expanded if misfolded protein is not able to acquire the final three-dimensional structure. For instance, prolonged interaction of BiP and GRP94 with the mutant, transport-incompetent form of Tg was shown on the mouse model of congenital goiter (26).

This principle was used to demonstrate physical interaction of the ER chaperones with their substrates using immobilized denatured proteins as a bait (15). Reproduction of the same experimental conditions allowed us to "fish out" ERp29 along with BiP and PDI from the FRTL-5 cell lysate or purified luminal fraction of the rat liver microsomes. At the same time, certain differences were noted in the ERp29 interaction with the substrate proteins as compared with BiP or PDI: 1) Unlike BiP, ERp29 could not dissociate from the Tg-Sepharose upon ATP elution, which indicates the absence of ATPase activity characteristic for many chaperones. A similar lack of any ATP effect was observed also in the co-immunoprecipitation experiments (see "Results"). 2) ERp29 appears to have narrower substrate specificity as compared with BiP and PDI. This was exemplified by the absence of binding to the denatured histone-Sepharose. 3) Despite the preferential association with the denatured Tg, appreciable amounts of ERp29 have been eluted also from the native ligand, which is in clear contrast to the BiP-like discrimination between properly folded and unfolded substrates and binding almost exclusively to the former. Taken together, these data indicate a unique mode of interaction of ERp29 with the substrate protein(s), which is more reminiscent of features of the chaperones with more narrow substrate specificity, such as GRP94 (27) or even dedicated escort chaperones, such as low density lipoprotein receptor-associated protein (28). The latter remains attached to the receptor even after the completion of its folding and further escorts it to the cell surface. Interestingly, the interaction of receptor-associated protein with Tg has been recently demonstrated both *in vitro* and *in vivo* (29). A similar role of the dedicated chaperone was proposed also for the *Drosophila* analog of ERp29, Windbeutel (10, 30).

Although luminal ER chaperones may leak from the ER, they are usually recycled back by the ERD2 receptor recognizing their KDEL (and similar) C-terminal sequence (31). Despite the existence of such retrieval mechanism, many luminal proteins, such as GRP94, PDI, calreticulin, BiP, and PDI, have been found in the cell exterior (22–25), and several reports have proposed possible functional significance of such localization (25, 32). The instances of the unusual localization of PDI are of particular interest for this study. ERp29 is a structural homo-

which was exchanged to the fresh medium, and aliquots representing equal amounts of cells were collected at the indicated time points, concentrated, and immunoblotted. 10 $\mu\text{g}/\text{ml}$ of BFA was added with the fresh medium. *C*, results of the densitometric analysis of immunoreactive bands ($n = 4$). Black bars, protein amounts related to the corresponding amounts at 1 h. Gray bars, protein amounts in the presence of BFA related to the corresponding amounts at 1 h without BFA.

log of PDI despite the absence of the active site motif, and because PDI may function also as a general chaperone, it was proposed that these two proteins might have some overlapping functions (9). However, extracellular function of PDI may differ from the ERp29 role, because PDI was found on the surface of FRTL-5 cells and proposed to react with Tg under acidic conditions (25), whereas our studies in living cells failed to reveal any cell surface expression of ERp29 (results not shown).

Possible reasons as to how KDEL proteins may escape recycling include saturation of KDEL receptor, defects in the retention system, and proteolytic removal of the C terminus bearing the retrieval signal (23, 32). It has been previously shown that overexpression of PDI does not result in the increased secretion of other KDEL proteins (33). In our study, substantial amounts of ERp29 and PDI were secreted even from the quiescent cells (Fig. 6). Consequently, the saturation of recycling mechanisms is not likely to be responsible for the ERp29 export. Electrophoretic migration of the secreted ERp29 was identical to the migration of the intracellular species, suggesting similar molecular masses. Extracellular ERp29 was also recognized by ERp29 antibodies, which were raised against a peptide from the C-terminal fragment of ERp29 (3) (results not shown). These data speak in favor of the intact C terminus of ERp29, available for ERD2 receptor. At the same time, BFA-regulated export of ERp29 and co-localization of Tg and ERp29 in the putative transport structures indicate that ERp29 may proceed via the secretory pathway in a manner typical for secretory proteins. It was suggested that in certain cases secretion of KDEL proteins could be triggered by shielding their C-terminal recognition sequence by the substrate secretory proteins (24). Based on the evidence presented in this study one can speculate that ERp29 export could occur because of such association with Tg. Such "co-secretion" is characteristic for so called escort chaperones (34) or molecular escorts (35) such as receptor-associated protein. Interestingly, the ERp29 analog in *Drosophila*, Windbeutel, was identified as such dedicated escort chaperone, shown to facilitate the transport of its substrate, Pipe, a putative oligosaccharide-modifying enzyme essential for embryonic development, to the Golgi (30).

However, despite the demonstrated co-localization of ERp29 and Tg in the cell exterior (Fig. 6A), we failed to detect ERp29/Tg association in the FRTL-5 cell medium utilizing immunoprecipitation approach (results not shown). Similarly, neither BiP nor GRP94 were found in the Tg or ERp29 immunocomplexes. It is worth noting that a similar absence of co-immunoprecipitating Tg, PDI, and BiP has been shown previously (25). Nevertheless, because the fate of Tg exported from the cultivated thyrocytes is quite different from the situation in the thyroid gland where secreted Tg is stored in the follicular lumen in form of compact, cross-linked aggregates (36), one cannot exclude the existence of the extracellular ERp29/Tg complexes *in vivo*.

Evidence provided in this work including co-immunoprecipitation studies, sucrose density fractionation, immunofluorescent co-localization of ERp29 and Tg, and, finally, affinity isolation of ERp29 on the denatured protein matrix unambiguously point to the specific association(s) of ERp29 within the multicomponent Tg folding complex in the ER. Al-

though it is tempting to speculate that ERp29 directly interacts with Tg in a chaperone-like manner, one cannot rule out its primary association with the other member(s) of the complex. We have shown, for instance, that BiP may be co-immunoprecipitated with ERp29 from the rat hepatoma cells (3). However, identification of two potential unfolded protein-binding sites in our recent study (9) may speak in favor of the direct ERp29/Tg interactions. Given the widespread pattern of ERp29 expression, similar interactions are anticipated also with the other secretory proteins, especially in the secretory cells with high levels of ERp29.

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