Protein-disulfide Isomerase (PDI) in FRTL5 Cells

pH-DEPENDENT THYROGLOBULIN/PDI INTERACTIONS DETERMINE A NOVEL PDI FUNCTION IN THE POST-ENDOPLASMIC RETICULUM OF THYROCYTES*

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Thyroglobulin (TG) is secreted by the thyrocytes into the follicular lumen of the thyroid. After maturation and hormone formation, TG is endocytosed and delivered to lysosomes. Quality control mechanisms may occur during this bidirectional traffic since 1) several molecular chaperones are cosecreted with TG in vivo, and 2) lysosomal targeting of immature TG is thought to be prevented via the interaction, in acidic conditions, between the Ser789–Met1172 TG hormonogenic domain (BD) and an unidentified membrane receptor. We investigated the secretion and cell surface expression of PDI and other chaperones in the FRTL5 thyroid cell line, and then studied the characteristics of the interaction between TG and PDI. We demonstrated that PDI, but also other chaperones such as calnexin and KDEL-containing proteins are exposed at the cell surface. We observed on living cells or membrane preparations that PDI specifically binds TG in acidic conditions, and that only BD is involved in binding. Surface plasmon resonance analysis of TG/PDI interactions indicated: 1) that PDI bound TG but only in acidic conditions, and that it preferentially recognized immature molecules, and 2) BD is involved in binding even if cysteine-rich modules are deleted. The notion that PDI acts as an “escort” for immature TG in acidic post-endoplasmic reticulum compartments is discussed.

The functional unit of the thyroid gland is the follicle, which is composed of a monolayer of epithelial cells, the thyrocytes, surrounding a closed space, the follicular lumen. The thyroid prohormone, thyroglobulin (TG), is synthesized by thyrocytes and then secreted into the follicular lumen in which the thyroid hormones themselves are synthesized. Hormonal secretion requires the endocytosis of mature TG (i.e. TG bearing hormone residues), its targeting to lysosomes, and the subsequent release of hormones. Each step of this bidirectional trafficking of TG is subject to regulation, in some cases mediated by molecular chaperones (1–3).

Thyroglobulins synthesize large amounts of TG (up to 50% of protein synthesis in the gland; Ref. 4). Glycosylation and chaperone-assisted folding of TG monomers (M, 330,000) take place in the endoplasmic reticulum (ER), and involve several chaperones including calnexin, BiP, ERp72, grp94, and grp170 (5–9). The dimerization of monomers, a process thought to abolish the interactions between secreted molecules and chaperones, probably determines the sorting and targeting post-ER of glycoproteins (10). The transit through the Golgi apparatus of newly synthesized TG leads to the modification of some high mannose type N-glycans into N-acetyllactosamine chains. Secreted glycoproteins have an almost unique property, mediated by an unknown regulatory mechanism, in that some of these lactosamine chains of TG are incompletely processed and have accessible GlcNAc moieties (11, 12). TG molecules are then targeted to small secretory vesicles and released via the regulat- ed secretory pathway of thyrocytes (13, 14).

In the follicular lumen, the prohormone undergoes several posttranslational modifications to produce thyroid hormones. These modifications include the iodination of tyrosyl residues, and the oxidative coupling of some of these residues to form triiodothyronine (T3) and tetraiodothyronine (thyroxine or T4) (1, 2). They also include the de novo formation of disulfide bridges, the multimerization of some TG molecules (15), and the progressive completion of their incompletely processed lactosamine N-glycans by the addition of galactose and sialic acid sugar moieties (12).

As TG molecules in the follicular lumen are very heterogeneous in terms of iodine and hormone content, it has been assumed that there is a mechanism that either preferentially targets iodine-rich molecules to lysosomes or prevents the catalysis of iodine-poor molecules. It has recently been reported that megalin, a 330-kDa glycoprotein associated with the cell surface, participates, at least partly, in TG internalization in some some thyroid cell preparations (16, 17). However, further studies are required to determine whether this process is selective for a given TG subpopulation, and no receptor specific for mature thyroglobulin has yet been shown to be responsible for the selective internalization of TG. The notion of an intrafollicular retention process for immature molecules arose from three main findings: 1) there exists, in vivo in the rat, a major recycling pathway from internalized TG back to the lumen (18); 2) during TG maturation, galactose and sialic acid are incorporated into some glycans (12), by a process known to occur in the trans compartments of the Golgi complex and the trans-Golgi network (19), and 3) immature TG preferentially binds a membrane receptor in acidic conditions, suggesting that there...
may be a means of recycling TG after endocytosis, in acidic compartments such as the endosomes and prelysosomes (20, 21). The receptor involved in binding has not yet identified, but the receptor-binding domain of TG has been located within the hormonogenic domain (Ser789-Met1178) of TG, which includes tyrosine residues involved in hormone formation and a cysteine-rich type 1 module (22).

Recent reports have established that in secretory cells some chaperones are secreted or cell surface-associated. For example, calnexin as well as many other “ER resident proteins” are present at the surface of thyocytes (23), and grp94 is secreted by pancreatic cells (24, 25). The best documented example is the protein-disulfide isomerase (PDI). PDI (M, 57,000) is an abundant soluble cellular protein of the secretory pathway (0.4% of total liver protein, Refs. 26–28) found on the cell surface of numerous cell types such as B cells (29, 30), platelets (31, 32), pancreatic cells (33), and hepatocytes (34). PDI may be secreted despite possessing the KDEL retention signal (34, 35).

It remains fully active and may be involved in structural modification of (glyco)proteins in the extracellular medium. Indeed, PDI catalyzes thiol-disulfide interchanges that may result in the rearrangement of protein-disulfide bonds. This is why cell-surface PDI is involved in the reduction of the disulfide-linked diptheria toxin heterodimer (36), cell surface events triggering the entry of the human immunodeficiency virus into lymphoid cells (37), shedding of the human thrytropin receptor ectodomain (38), cell surface recognition during neuronal differentiation of the retina (39), and, more generally, controls the redox state of existing exofacial protein thios or reactive disulfide bonds (40). PDI, and some other chaperones such as BiP and grp94, are secreted with TG in vivo into conditions into the follicular lumen (41, 42), suggesting that these chaperones may be involved in the structural modification of the prohormone in hormone biogenesis.

We investigated the secretion and cell surface expression of PDI and other chaperones in the FRTL5 (thyroid) cell line, and then studied the characteristics of the interaction between TG or derived fusion proteins and PDI in FRTL5 cells. We report two key observations: 1) PDI is secreted and associated with membranes, which implies that it may be present in vivo in post-ER compartments; and 2) PDI interacts with the membrane-binding domain of TG in acidic compartments. The potential implications of these observations are discussed.

MATERIALS AND METHODS

Chemicals

Amplify, [35S]methionine/cysteine, NaCl, protein A-Sepharose, and PD10 columns were obtained from Amersham Pharmacia Biotech; 10-DOGEN, bovine protein-disulfide isomerase, fluorescein isothiocyanate (FITC), Sulfo-SHPP, and chemiluminescence kits and reagents (SuperSignal, Duogen, bovine protein-disulfide isomerase, fluorescein isothiocyanate (FITC), Sulfo-SHPP, and chemiluminescence kits and reagents (SuperSignal, Duogen, Duogen, Duogen). The labeling medium was removed and replaced with Couen’s modified Ham’s F-12 medium, and the cells were incubated for various lengths of time (the chase period). The media were removed after the chase period, and, protease inhibitors were added (1 mM EDTA, 1 mM phenylmethanesulfon fluoride, and 10 μg/ml each leupeptin and pepstatin) and the mixture centrifuged (1 h at 100,000 g). For treatment of the cells with colchicine or brefeldin A, cells were incubated in Couen’s medium containing either 40 μM colchicine or 20 μM brefeldin A for 30 min before labeling and the chase period in medium containing the same concentrations of these drugs. The cells were washed four times with ice-cold PBS and harvested by scraping. They were then centrifuged at 1,500 revolutions/min for 10 min, suspended in 10 ml of ice-cold PBS and recentrifuged. The resulting cell pellets were suspended in 1 ml of lysis buffer (10 ml Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40). The cell lysates were centrifuged at 12,000 × g for 10 min to remove cell debris. The resulting supernatants were cleared by centrifugation with 20 μl of protein A-Sepharose and 20 μl of a 50% suspension of Protein A-Sepharose for 1 h at 4 °C. The suspension was centrifuged at 12,000 × g for 3 min, the supernatant discarded, and the pellet washed four times with lysis buffer and once with PBS. The immunoprecipitate was suspended in SDS-PAGE sample buffer containing 2% SDS with or without 1% β-mercaptoethanol and subjected to SDS-PAGE. Gels were dried by conventional methods, and radiolabeled bands were detected by phosphorimaging (Molecular Dynamics, Inc., Sunnyvale, CA). The leakage of proteins from injured cells was assayed by determining lactate dehydrogenase activity.

Immunodetection of Cell Surface PDI and Chaperones

FRTL5 cells were grown in Lab Tek I chambers, washed briefly in PBS, and mixed in 2% formaldehyde at room temperature for 15 min. The cells were then washed twice in PBS and incubated with blocking buffer (PBS containing 1% BSA) for 10 min at room temperature in a humidified chamber. The cells were mixed directly, without washing, with the primary antibody in 0.1% BSA in PBS, and the mixture was incubated for 1 h. The cells were then washed five times with 0.1% BSA in PBS, and were incubated for 1 h with the fluorochrome-conjugated secondary antibody in 0.1% BSA in PBS. The cells were thoroughly washed in PBS, mounted on slides in Moviol, and viewed with a confocal microscope (Leitz DMR BE, Leica TSC 4D, Heidelberg, Germany).

Quantitation of PDI Secretion

We determined the level of PDI secretion, by subjecting serially diluted (standardized based on protein content) samples of culture supernatant to SDS-PAGE and fluorography.
medium to slot blot analysis on nitrocellulose membranes. The signal obtained was compared with that given by known amounts of purified PDI. Blots were probed with anti-PDI antibody, treated for chemiluminescence, scanned, and analyzed with a PhosphorImager and the ImageQuant software package (Molecular Dynamics).

Radiolabeling

Sulfos-SHPP was iodinated according to the manufacturer’s instructions. Briefly, 1 ml of 125I-Na was added to 10 µl of a freshly prepared solution of sulfo-SHPP in dimethyl sulfoxide (0.1 mg/ml). The following were then added sequentially: 10 µl of chloramine T (5 mg/ml), 100 µl of hydroxyphenyl acetic acid (1 mg/ml), and 10 µl of sodium metabisulfite (12 mg/ml). 125I-Labeled sulfo-SHPP (200 µCi) was incubated in 25-cm3 flasks with confluent FRTL5 cells, previously washed three times with ice-cold PBS for 1 h each at 2–4 °C. The labeling medium was removed, and the cells were washed with PBS at 4 °C and recovered by scraping into ice-cold PBS. The cells were washed, and cell lysates were obtained for immunoprecipitation of PDI as described above.

PDI (10 µg) was iodinated by incubation with IODOREN and 125I-Na in 50 µl of 50 mM Tris-HCl buffer, pH 8.0, for 15 min at 4 °C. The reaction was stopped by adding 1 ml of 10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 20 mM KI. Iodinated molecules were isolated by gel filtration through PD10 columns and extensive dialysis. The same protocol was used for TG iodination, except that gel filtration was performed through Sephadex-200 columns (Amersham Pharmacia Biotech).

Construction of Human Thyroglobulin Fusion Peptides

**RNA Isolation**—Total RNA was extracted from the thyroid tissues of patients with Graves’ disease, as described by Chomczynski and Sacchi (43). Briefly, tissue (10 mg) was crushed in 3 ml of RNABlue solution (Eurobio). Chloroform (300 µl) was added, and the mixture was vigorously vortexed and centrifuged at 15,000 × g for 30 min. The aqueous phase was transferred to a microcentrifuge tube containing an equal volume of isopropanol. RNA was recovered by precipitation overnight at −20 °C and centrifugation at 12,000 × g for 20 min. The pellet was washed with 75% ethanol and suspended in diethyl pyrocarboxylate-treated water.

**Amplification by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**—The first strand cdNA was synthesized by incubation of 1–3 µg of total RNA with Moloney murine leukemia virus reverse transcriptase (5 units, Life Technologies, Inc.) for 1 h at 42 °C, in a volume of 30 µl. The resulting first strand cdNA was then used for PCR amplification.

The membrane receptor binding domain of TG (Ser269-Met178) was amplified using the following primers: the forward primer was 5′-GGGATCCGCGAGGCCACTCCAGGGTGTCG-3′ (2374–2394) and the reverse primer, 5′-CTCTAGAGAGCACATTTCAGAGGCTTGG-3′ (3494–3474; reverse). The cysteine-rich repeat unit 9 (RU9, according to Ref. 44) forms part of the binding domain, was amplified using the primers 5′-GATTCATTTTGGATTCCAGGCAGCCGGCGGG-3′ (3241–3261; forward) and 5′-CTCTAGAGAGCACATTTCAGGGTGTCG-3′ (3494–3474; reverse). Each of these primers contained a unique restriction site, BamHI for the forward primers and XhoI for the reverse primers. The expected protein sequences of the TG-derived fusion proteins included Pro103-Leu115 for the membrane-binding domain of TG and Leu116–Leu153 for the RU9 cysteine-rich motif.

**Cell Surface Labeling of PDI and TG-FITC**

Human or porcine TG was conjugated with fluorescein isothiocyanate (TG-FITC) as follows. TG (150 µl of a 1 mg/ml solution) was added to TG in 0.1 M carbonate buffer, pH 9.0 (20 mg in a 1.95-ml final volume), and the mixture was incubated in darkness for 1 h at room temperature. TG-FITC was recovered by gel filtration through a PD10 column (Amersham Pharmacia Biotech). It was dialyzed extensively, and aliquots (200 µl, 10^-3 µl final concentration) were stored at −20 °C. FRTL5 cells were grown in Lab Tek I chambers, washed briefly in PBS, and fixed in 2% formaldehyde at room temperature for 15 min. Cells were washed twice in PBS and incubated in blocking buffer (PBS containing 10% BSA) for 10 min at room temperature in a humidified chamber. For PDI or TG labeling, cells were fixed immediately, without washing, with primary antibodies in PBS containing 0.1% BSA, and incubated for 1 h. The cells were washed five times with PBS and once with 200 µl of PBS containing 2% bovine serum albumin. Thyroglobulin (5 x 10^10 to 10^14 cpm in a binding buffer consisting of 25 µM acetyl buffer, pH 5.0, 150 mM NaCl, 5 mM CaCl2, and 0.1% BSA) was added and incubated alone or in the presence of competitors at 4 °C for 90 min. The wells were washed four times with the binding buffer, and radioactivity was counted. The results are means of at least three separate experiments performed in duplicate or triplicate.

Chaperones detected in membrane preparations were determined by immunoblotting with microtiter plates coated with membranes (10 µg/well). The plates were coated by incubation for 2 h at 37 °C. The plates were washed with PBS and incubated for 2 h with PBS containing 3% skim milk powder. They were then incubated with monoclonal antibodies directed against chaperones (1,500 dilution). Bound antibodies were detected by chemiluminescence using horseradish peroxidase-linked anti-mouse IgG (1/10,000 dilution), extensive washing and enzymatic reaction with OPD (Dako). Absorbance was read at 490 nm on an ELMER Reader MF (Labsystem).

**Production and Purification of Fusion Proteins**

Bacterial clones containing the various constructs were cultured overnight in 50 ml of LB medium at 37 °C with shaking. An aliquot (50 µl) of the overnight culture was used to inoculate 1 liter of LB, which was grown to an optical density of 0.5 at 600 nm of the culture reached 0.4 (i.e. 2 x 10^7 cells/ml). Synthesis of the fusion proteins was induced by adding isopropyl-1-thio-β-galactopyranoside to a final concentration of 0.1 mM and incubating at 32 °C for 90–120 min. The culture was then centrifuged at 1500 × g for 10 min. The bacterial pellet was suspended in 50 ml of lysis buffer (10 mM phosphate buffer, pH 7.0, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 10 mM EGTA) and placed in a freezer at −20 °C overnight. The lysate was thawed at 4 °C, in the presence of 1 mM PMSF and subjected to sonication with 1-min pulses for 20 min. NaCl (final concentration 0.5 M) was added and the mixture centrifuged at 40,000 × g for 30 min at 4 °C.

The fusion proteins were purified on amylose resin, according to the manufacturer’s instructions. The protein-containing fractions were dialyzed and concentrated (1 mg/ml final concentration) against phosphate-buffered saline (PBS) in a MicroPordicon apparatus (BioMolecular Dynamics). The level of purification of these fusion proteins was checked by SDS-PAGE and immunoblotting with anti-maltose-binding protein (MBP) and anti-TG antibodies.

**Membrane Preparation and Binding Assay—**FRTL5 plasma membranes were prepared as described previously (5, 6). Solid-phase assays of TG and fusion peptide binding to FRTL5 membranes were performed according to established methods (21, 22). Membranes were added (20 µg of total protein in 40 µl of PBS buffer) in the flatchanged bottom wells of a microtiter plate (Falcon 3911, Becton Dickinson, Oxnard, CA) and were incubated for 1 h at 37 °C and then overnight at 4 °C. The wells were washed three times with 150 µl of PBS and once with 200 µl of PBS containing 2% bovine serum albumin. Thyroglobulin (5 x 10^10 to 10^14 cpm in a binding buffer consisting of 25 µM acetyl buffer, pH 5.0, 150 mM NaCl, 5 mM CaCl2, and 0.1% BSA) was added and incubated alone or in the presence of competitors at 4 °C for 90 min. The wells were washed four times with the binding buffer, and radioactivity was counted. The results are means of at least three separate experiments performed in duplicate or triplicate.

Cloning and Sequencing of PCR Products—PCR products were purified from agarose gel and cut with BamHI and XhoI endonucleases. They were then ligated into the BamHI/XhoI-cleaved PMal-CR1 expression vector (Biolab) and used to transform Escherichia coli TB1. The TG fragment inserts of the plasmids of positive clones were checked by sequencing. The DNA was sequenced using M13 (−47) and reverse M13 (−48) primers (New England Biolabs). We used the cycle sequencing method and the Thiodamin Terminator Cycle Sequencing Kit (Applied Biosystems/Perkin-Elmer). Sequences were determined with an ABI Prism 310 and were aligned with the Tg cdNA sequence using Autoassembler software (Applied Biosystems/Perkin-Elmer).

RT-PCR generated two cdNA fragments, one with the expected sequence, based on the wild-type mRNA (43, 44), and the other with exons 13, 14 and 15 deleted. Exons 14 and 15 encode the cysteine-rich R9U motif (His1295-Cys1300). This finding is novel, and we therefore tried to determine the incidence of this deletion in various physiopathological conditions. The results of this investigation will be published elsewhere. A. Mezghrani, J. Courageot, J. C. Mani, M. Pugniere, P. Bastiani, and R. Miquelis, manuscript in preparation.
proteins bands only (Fig. 1).

Reprobed with a monoclonal anti-PDI antibody (clone RL90).

detected (Fig. 1).

Regenerated by washing with 10 mM NaOH, pH 11.8, and equilibration with 10 mM Tris/HCl, pH 7.0. Protein were separated by SDS-PAGE in a 10% acrylamide gel, transferred to nitrocellulose membranes, probed with a monoclonal anti-PDI antibody detected by chemiluminescence, as described under "Materials and Methods." Molecular masses are in kDa.
eoscent probe (0.5 × 10−7 m) in 10 mM acetate buffer, pH 5.0, 0.15 M NaCl, for 1 h at 4°C. They were washed with acetate buffer and fixed in 2% formaldehyde. They were then mounted on slides in Mowiol and viewed with a confocal microscope as follows.

Cell samples were examined using a Leica TCS 4D invert confocal microscope with argon/krypton multilasers giving excitation lines at 568 and 488 nm, respectively. The data from the channels were collected simultaneously, using the narrow bandpass filter setting built into the instrument. Double labeling experiments usually employed a combination of FITC and Texas Red dyes (secondary antibodies) to eliminate channel overlap effects. Specimen were tested for overlap by turning off individual laser lines while continuously scanning the two channels. Data were collected with 8-fold averaging at a resolution of 512 × 512 pixels using optical slices of between 0.5 μm and 1 μm. The microscope was a Leitz DMIR BE utilizing a 100× oil immersion objective lens (numerical aperture 1.4). Data sets were processed using the Scanware software, then exported for preparation for printing using Photoshop. Data were projected onto a single plane, and the contrast was adjusted prior to export as TIFF files to Adobe Photoshop.

Surface Plasmon Resonance Analysis of the TG/PDI Interaction

A BIAcore biosensor system (BIAcore AB, Upsalla, Sweden) was used to study the kinetics of TG binding to immobilized PDI. All experiments were performed at 25 °C. The sensor chip was washed with 10 mM Hepes, pH 7.5, 150 mM NaCl, 0.005% BIAcore surfactant P20 (buffer A) (BIAcore AB) between injections. Purified PDI (100 μg/ml) was directly bound, via its amino groups, to the sensor surface (CM5 sensor chip), activated by fluorescent probe (0.5 μM) in 2% formaldehyde. They were then mounted on slides in Mowiol and viewed with a confocal microscope as follows.

RESULTS

Detection and Determination of the PDI Content of FRTL5 Cells—In Western blots of proteins isolated from CEM, BHK21, and FRTL5 cells, probed with the anti-PDI IgG raised against the whole molecule, a band of Mr 63,000, was detected. In some experiments, an additional band of Mr 48,000 to 51,000 was also detected (Fig. 1A). The celluose membrane was stripped and reprobed with a monoclonal anti-PDI antibody (clone RL90). This resulted in the detection of the Mr 57,000 and Mr 51,000 bands only (Fig. 1B), demonstrating that: (i) the Mr 63,000 band was not related to PDI and (ii) the additional Mr 51,000 band was probably a product of PDI degradation. This was confirmed by the subsequent finding that the 51-kDa protein was produced after incubation of a freshly prepared FRTL5 homogenate (Fig. 2, lane 1) for 30 min at 37 °C or at room temperature (Fig. 2, lanes 2 and 3, respectively).

Densitometry showed that there was more PDI in FRTL5 cells than in the other cell types studied. The relative amount of PDI present was determined by subjecting serially diluted cell lysate samples to slot blot analysis, probing with monoclonal antibodies, and comparing the signal obtained with that obtained using known amounts of purified PDI. The PDI concentrations in CEM cells has been estimated to be 0.42% (0.33–0.56%, n = 6), that of BHK21 cells, 0.78% (0.68–0.89%, n = 6) and that of FRTL5 cells to be 1.05% (0.85–1.25%, n = 6) of total protein in the homogenates.

PDI Is Secreted by FRTL5 Cells—We metabolically radiolabeled FRTL5 cells and recovered PDI by immunoprecipitation of cell lysates and media. Cellular PDI was recovered as two major protein bands corresponding to the monomer (57-kDa monomer) and its principal degradation product (51 kDa) (Fig. 3, left). PDI was detected in the medium after a 1-h chase period, and the amount present in the medium increased between 1 and 2 h (Fig. 3, right).

It is also possible to isolate PDI from a culture medium in which the cells have been radiolabeled at equilibrium for 24 h. Apart from the major 57-kDa protein, a 330-kDa protein was also precipitated (Fig. 4, lane 1). This protein was identified as the thyroglobulin monomer (Fig. 4, lanes 3 and 6) and is probably present as a contaminant (Fig. 4, lane 7). Indeed, the same pattern was observed, with similar amounts of PDI recovered, in non-reducing conditions (Fig. 4, lane 2), showing that most of the PDI and TG molecules were not covalently bound. This is further supported by the observation that PDI was not detected in immunoprecipitates of TG, with or without β-mercaptoethanol (Fig. 4, lanes 5 and 6). We also isolated BiP from the culture medium. Similar fractions were isolated in the presence and absence of ATP, throughout the immunoprecipitation experiments. This excludes the possibility of ATP-dependent binding between the secreted forms of BiP and TG (Fig. 4, lanes 3 and 4). It is interesting to note that BiP was recovered using a polyclonal antibody directed against the eight amino acid residues at the extreme C terminus of BiP, including the KDEL sequence.

PDI was probably secreted by FRTL5 cells rather than leaked from injured cells, because only 0.8% after 24 h, and 1.8% after 48 h, of total lactate dehydrogenase was released from FRTL5 cells, whereas densitometry showed that 7.5% of...
total PDI was present in the medium (i.e. the medium + cell extracts) after 24 h. To confirm that PDI recovered from the media was secreted and not result from cell rupture, we treated cells with either brefeldin A or colchicine prior to and during the labeling and chase periods. Densitometric analysis of fluorographs of PDI immunoprecipitates showed that the amount of newly synthesized PDI in cells was slightly increased after drug treatment (control: 0.048 ± 0.017; brefeldin A: 0.055 ± 0.018; colchicine: 0.054 ± 0.016; n = 3), whereas there was much less PDI in the media, with a reduction of 75–95% (control: 0.128 ± 0.016; brefeldin A: 0.022 ± 0.008; colchicine: 0.032 ± 0.006; n = 3).

**PDI Is Present at the Surface of FRTL5 Cells—** PDI was recovered by immunoprecipitation following the radioiodination of cell surface proteins. Similar amounts of PDI were recovered in the absence (Fig. 5, lane 2) and presence (Fig. 5, lane 3) of β-mercaptoethanol. This rules out disulfide bridge formation as the mechanism of PDI cell association at the cell surface. If the anti-KDEL antibody was used after the anti-PDI antibody, six proteins were immunoprecipitated from the labeled plasma membrane fraction (Fig. 5, lane 4) as reported previously in a study of exocrine pancreatic cells (39). These proteins, thought to include grp94 and BiP (39), are probably chaperones containing the KDEL sequence.

PDI was readily detected on the cell surface of FRTL5 cells by confocal microscopy (Fig. 6a). All cells were labeled. The labeling was punctate and covered the entire cell surface, although it was more dense in some regions than in others, suggesting the formation of aggregates or clusters. Calnexin was also detected but the labeling pattern differed between cells. The most strongly labeled cells also showed punctate labeling (Fig. 6b). In contrast, despite numerous studies on different cell preparations, we failed to detect specific labeling of BiP at the cell surface (Fig. 6c). A possible explanation is that, whereas the subcellular distribution of BiP did not markedly differ from that of PDI (Fig. 6d) and calnexin (Fig. 6e) (all three chaperones presented the same punctate labeling pattern in vesicular structures located throughout the cytoplasm) the level of fluorescence staining was less intense for BiP (Fig. 6f). We concluded that, if present at the cell surface, BiP was not readily detectable using this approach, and we did not further investigate the localization of various chaperones by confocal microscopy.

As stated in the Introduction, TG is thought to interact with various molecules, either constituents of the plasma membrane (melanin, interaction at neutral pH) or molecules thought to pass via the surface of the cell during their intracellular transport (the putative TG receptor, interaction in acidic conditions). The cell membrane chaperones associate with misfolded (3, 5–9) and denatured TG (47). This raises the question as to whether these chaperones also associate with particular sub-populations of TG molecules in preparations that we use. To clarify this issue, we first tried to identify the locations of the putative receptors for TG and PDI at the cell surface (Fig. 7). In our conditions, endogenously expressed rat TG was not detected on the surface of FRTL5 cells, regardless of the type of anti-TG antibody used (monoclonal or polyclonal, data not shown). As deduced from biochemical studies of the TG binding characteristics on plasma membrane preparations (21, 22), cell surface-associated TG could only be detected after addition and incubation of exogenous TG in acidic conditions at 4 °C (data not shown). In the present study, we have used fluorescein isothiocyanate-conjugated TG to visualize directly the binding sites of TG on the cell surface. This binding was as specific as that observed on membrane preparations, since in control experiments we noted that it could be specifically prevented by preincubation with non-derivatized TG (10–5 M concentration, data not shown). Fig. 7 showed that PDI was readily detected on the surface of these FRTL5 control cells (Fig. 7a). TG-FITC did not bind to cells at neutral pH (Fig. 7b), but binding was abundant and clearly detectable in acidic conditions (Fig. 7c).
The detection in the same cellular preparation of TG bound to cells in acidic conditions (Fig. 7d) and of PDI (Fig. 7e) demonstrated that this receptor and PDI were located in the same plasma membrane domains (Fig. 7f). Thus, PDI is either located in the vicinity of the receptor or is itself the receptor that binds TG in acidic condition.

**PDI Is Involved in the Binding of TG to FRTL5 Membranes**—We have reported that the membrane receptor that binds TG in acidic conditions interacts with the various species of TG and preferentially binds molecules with a low iodine content (21). The domain of TG responsible for the binding of the molecule to membranes is located within the Ser789–Met1172 sequence (22). This domain contains two N-linked glycan moieties and a cysteine-rich motif encoded by exons 14 and 15. The cysteine-rich motif is not involved in the interaction (22). The glycan moieties are involved in the interaction because their enzymatic cleavage reduces binding affinity by an order of magnitude (21). Bearing these previous findings in mind, we assessed whether TG fusion proteins encoding all or part of the binding domain were fully active, and investigated whether PDI was involved in the recognition of this binding domain.

The binding activities of TG fusion proteins at pH 5.0 were analyzed using 125I-TG, TG and TG fusion proteins as competitors, and microwell plates coated with FRTL5 plasma membranes (Fig. 8). Unlike the control proteins, MBP and MBP-paramyosin, the TG-fusion protein containing the binding domain (Pro759-Leu1163 fragment, BD in Fig. 8) inhibited the interaction between 125I-TG and membranes. However, as expected for a non-glycosylated binding region (21), the binding affinity of this peptide was lower than that of TG itself ($K_{D}$ = 0.2 x 10$^{-8}$ M and 0.3 x 10$^{-7}$ M). The peptide fragment lacking RU9, due to the alternative splicing of exons 14 and 15 (BDΔRU9), had the same degree of inhibitory activity as BD.

We investigated whether PDI, alone or with other chaperones, was directly involved in this binding. We first identified the chaperones that were present in our preparations of plasma membranes (Fig. 8). Unlike the control proteins, MBP and MBP-paramyosin, the TG-fusion protein containing the binding domain (Pro759-Leu1163 fragment, BD in Fig. 8) inhibited the interaction between 125I-TG and membranes. However, as expected for a non-glycosylated binding region (21), the binding affinity of this peptide was lower than that of TG itself ($K_{D}$ = 0.2 x 10$^{-8}$ M and 0.3 x 10$^{-7}$ M). The peptide fragment lacking RU9, due to the alternative splicing of exons 14 and 15 (BDΔRU9), had the same degree of inhibitory activity as BD.

We investigated whether PDI, alone or with other chaperones, was directly involved in this binding. We first identified the chaperones that were present in our preparations of plasma membranes. We found, as observed previously (Fig. 5, lane 4; Fig. 6b) that in addition to PDI, other chaperones such as calnexin, grp94, and BiP were present in these preparations (Fig. 9, left). Monoclonal antibodies directed against PDI inhib-
ited TG binding to these membrane preparations, confirming that PDI is either in the vicinity of the receptor or is directly involved in TG binding to membranes (Fig. 9, right). In contrast, monoclonal antibodies directed against grp94, BiP, and calnexin had no effect on the binding of TG (Fig. 9, right), demonstrating that none of these chaperones were involved in binding. TG binding was strongly inhibited (80%) at a concentration of 1 μg/ml, by a monoclonal antibody with an epitope close to one of the thioredoxin sites (monoclonal antibody RL90) and was inhibited by about 30% by a monoclonal antibody directed against the C-terminal domain of PDI (Fig. 9, right).

We then used a biochemical approach to demonstrate that PDI is directly involved in the binding of TG to the membrane (Fig. 10). The surface proteins of FRTL5 cells were radiolabeled with 125I. The cells were lysed, and the cell lysates were incubated, in various conditions, with TG adsorbed onto agarose beads. If the agarose beads were incubated and washed in neutral pH conditions, the material associated with the beads gave four major bands in SDS-PAGE. Three of these bands had molecular masses below 26.6 kDa. The fourth had a molecular mass of about 57 kDa (Fig. 10, lane 1), identical to that of the 125I-labeled PDI used as a standard (Fig. 10, lane 5). If the agarose beads were incubated with cell lysate in acidic conditions (pH 5.0), even with an aliquot one-fifth the volume of that used in neutral conditions, a larger amount of the 57-kDa protein was recovered and the low molecular weight contaminating bands were not detected (Fig. 10, lane 2). The 57-kDa band was not recovered if the lysate was cleared by an anti-PDI monoclonal antibody prior to the experiment (Fig. 10, lane 3). Therefore, PDI was the only radioiodinated cell membrane protein that bound TG in acidic conditions.

Surface Plasmon Resonance Analysis of the TG:PDI Interaction—Finally, we investigated whether PDI was itself sufficient for both TG binding and the selection of immature mole-

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**Fig. 8.** Inhibition of the binding of 125I-TG to thyroid membranes coated on multwell microtiter plates by TG-derived fusion proteins. The binding of labeled porcine TG to membranes was challenged with human TG, porcine TG, MBP, the TG-derived MBP fusion proteins BD (BD is the membrane-binding domain of human TG (Pro729–Leu1163)), BDARU9 (BDARU9 is that of the human TG variant with the cysteine-rich module RU9 (ΔHis1025–Cys1160) deleted), and RU9 (RU9 is the RU9 cysteine-rich module (Leu1080–Leu1163)). MBP and MBP-paramyosin were used as controls.

**Fig. 9.** Inhibition of the binding to FRTL5 membranes of 125I-TG or TG-derived fusion proteins by anti-PDI antibodies. Left, membranes (10 μg) were used to coat multwell microtiter plates, and chaperones were detected by probing with antibodies and revealed, the primary antibodies then being detected by enzymatic reaction using horseradish peroxidase-linked anti-mouse IgGs as described under “Materials and Methods.” A monoclonal anti-β-galactosidase was used as control. Right, membranes (20 μg) were coated on multwell microtiter plates and the binding of 125I-labeled human TG or fusion proteins was challenged using anti-grp94, BiP, calnexin, and PDI monoclonal antibodies (1 μg/ml). The results shown are those of three experiments performed in duplicate.

**Fig. 10.** Isolation and SDS-PAGE analysis of the FRTL5 cell surface TG-binding protein. FRTL5 cells were cell surface-labeled and lysed as described under “Materials and Methods.” The lysate was aliquoted. Aliquots were incubated and washed in the presence of 250 μl of TG-agarose beads in: 1.50 ml of lysate in PBS, pH 7.2 (lane 1); 0.3 ml of lysate in acetate buffer pH 5.0 (lane 2); 0.3 ml of lysate cleared by a monoclonal anti-PDI antibody (clone RL90) prior to use (lane 3). Lanes 4 and 5 correspond to aliquots of total cell surface iodinated proteins and 125I-PDI, respectively. Proteins were resolved by electrophoresis in a 10% acrylamide gel in the presence of β-mercaptoethanol. Molecular masses in kDa are shown.
cules, via binding to the binding domain. Real time studies of
TG binding to immobilized PDI were recorded on a biosensor
using PDI as a ligand and various sources of TG as analytes. No
binding occurred at neutral pH, but binding did occur in acidic
conditions (data not shown). We decided to analyze the inter-
actions at pH 5.0 to enable us to compare the results to those
obtained previously. We found that TG from pigs and human
of membrane receptor binding domain. 3) Surface plas-
mon resonance analysis of TG/PDI interaction characteristics
was detected with either RU9 or MBP (data not shown).

factor of 3:

\[ K_D = \frac{k_o}{k_d} \]

and 2.1 \( \times 10^{-8} \) M, respectively, see Table I). About three times
as much iodine-poor TG (iTG, iodine content
was 0.1%) as normal
TG (TG, iodine content = 1%) bound to PDI (Fig. 11). The binding affinity for iodine-poor TG was also higher than that
for normal TG (\( K_D = 1.0 \times 10^{-9} \) M and 1.6 \( \times 10^{-9} \) M, respect-
ively, Table I). Thus, these results are consistent with previ-
ous findings for membrane preparations indicating: 1) that TG
molecules are heterogeneous and only a subset is capable of
binding, and 2) that modifications clearly correlated with matura-
tion affect the binding activity. We found that fusion pro-
teins encoding the binding domain also bound to PDI. BD
bound to PDI with an affinity one-tenth that of native TGs (\( K_D =
3.8 \times 10^{-8} \)), as expected for the non glycosylated binding
domain (Fig. 8; see also Fig. 4 in Ref. 21). Deletion of the type
I cysteine-rich module did not reduce this binding (it even
seemed, for still unknown reasons, to increase binding by a
factor of 3: \( K_D = 1.1 \times 10^{-7} \)). No surface plasmon resonance
signal was detected with either RU9 or MBP (data not shown).

**DISCUSSION**

We report the following novel findings. 1) PDI is secreted by
and associated with the surface of FRTL5 thyroid cells. 2) The
binding of TG to FRTL5 cell membranes in acidic conditions is
selectively inhibited by anti-PDI antibodies. These antibodies
also inhibit the binding of fusion proteins encoding the Ser
Met membrane receptor binding domain. 3) Surface plas-
mon resonance analysis of TG/PDI interaction characteristics
indicated: (i) that PDI binds TG, but only in acidic conditions,
and that it preferentially recognizes immature molecules, and
(ii) that the Ser Met domain is involved in binding, even if
the cysteine-rich module is deleted. These results, and the
potential function of PDI as a binder of secreted TG molecules
in acidic post-ER compartments, are of particular importance.

FRTL5 thyroid cells are an additional secretory cell type in
which some molecular chaperones can escape the ER compart-
ment, even if they possess the C-terminal KDEL retention
signal (33, 34). The mechanism of secretion by the various cell
types is unknown. Secretion may be due to the high level of
chaperones in the ER of these cells, or may be an overflow of the

KDEL or other ER retention mechanisms (48, 49). However, the
specific overproduction of PDI in CHO cells (50) or HT 1080
fibrosarcoma cells (40) increases the secretion of PDI but not
other resident endoplasmic reticulum peroteins containing the
KDEL-sequence, such as grp78 and grp94, suggesting that
“PDI secretion and cell-localization is a specific event” (40),
probably related to a specific function.

PDI has been implicated in the reduction of proteins present
at, or interacting with, the cell surface, including throm-
bospondin (51), plasmin (52), diptheria toxin (36), and
the human immunodeficiency virus type I envelope glycoprotein
(37). More recently, it has been reported that secreted PDI
manipulates the redox state of exofacial extracellular protein
thiols/disulfides, thereby controlling the function of extracellu-
lar proteins (40). It is therefore possible that in vivo PDI is
involved in the structural modification of TG, particularly the
formation of disulfide bridges between and within chains, that
takes place in the follicular lumen during the synthesis of
thyroid hormones (15).

We quantified PDI secretion by FRTL5 cells and found that
it was as high as 15.01 ± 3.71 \( \mu \)g/ml of PDI/5 ml of culture medium
per 48 h (range: 18.7–13.1, \( n = 4 \)) for semiconfluent cells. This
estimation was made in a cell line known to produce large
amount of secreted molecules, at least in the case of TG. Fur-
ther studies are therefore required to compare the secretion of
PDI in thyroid cells, primary cultures, and other cell lines. 2
Nonetheless, the quantity outside the ER appears to be far
negligible, and PDI may therefore be involved in TG
metabolism in the follicular lumen in vivo. Studies in vitro have
shown that PDI has two distinctive features independent of its
function as a catalyst; at high concentration, it acts as a chaper-
one, inhibiting aggregation, but at low concentration, it fa-
cilitates aggregation (anti-chaperone behavior) (28, 53). One
example of possible anti-chaperone behavior by PDI is the
small quantities of PDI molecules that have been found asso-
ciated with highly cross-linked aggregates in the follicular lu-
men (41). These aggregates are 50–500 \( \mu \)m in size, may reach
concentrations of 590 mg/ml, and consist of TG molecules with
high iodine but no hormone (41). The formation of these aggre-
gates, which are also known as “colloid globules” (41), therefore
prevents the homing of misfolded and hormone-poor prohor-
mones to the endocytotic secretory pathway of thyrocytes.

However, our results suggested a novel function for PDI,
different from those previously described. First, the interaction
between TG and PDI occurs in acidic conditions. For reasons
explained elsewhere (20, 21), we decided to analyze this inter-
action at pH 5.0. However, it also occurs to a significant extent
at pH 6.0, and at pH 5.75 fixation was about 30% the maximum
(20, 21), which suggests that it may take place in vivo, in any of
the acidic compartments of the cellular exocytosis (54–56) and
endocytosis (57–59) pathways. Second, in contrast to the situ-

**TABLE I**

| TG or TG fragments | \( k_o \) | \( k_d \) | \( K_D \) |
|-------------------|---------|---------|---------|
| hTG               | \( 1.9 \times 10^{4} \) | \( 3.0 \times 10^{-4} \) | \( 1.6 \times 10^{-9} \) |
| iktG              | \( 2.6 \times 10^{3} \) | \( 2.6 \times 10^{-4} \) | \( 1.0 \times 10^{-9} \) |
| pTG               | \( 2.7 \times 10^{4} \) | \( 5.6 \times 10^{-4} \) | \( 2.1 \times 10^{-8} \) |
| BDARU9            | \( 2.4 \times 10^{4} \) | \( 2.6 \times 10^{-4} \) | \( 1.1 \times 10^{-8} \) |
| BD                | \( 9.2 \times 10^{3} \) | \( 3.5 \times 10^{-4} \) | \( 3.8 \times 10^{-8} \) |

**Fig. 11.** Typical sensorgrams (response in arbitrary units (RU) versus time in s) of TG binding to PDI (50 \( \mu \)g/ml in acetate buffer, pH 5.0). Curve 1, normal human TG (hTG); curve 5, iodine-poor human TG (iTG); curve 3, porcine TG (pTG); curve 4, TG fragment BD; curve 2, TG fragment BDARU9.
tyrosine residues involved in iodonation and hormone formation (22); (2) the binding domain is located in the N-terminal region of TG, which is highly cross-linked via 11 repeated cysteine-rich motifs and undergoes profound structural changes during its maturation in the follicular lumen (46); and (3) iodonation and hormonogenesis are thought to occur after excocytosis, at the cell surface, when TG comes into contact with thyroxoperoxidase. We are investigating this possibility further in two ways. First, as PDI is not an integral membrane protein, we are trying to identify the partners responsible for its association with the membrane and to determine whether and how PDI is transported with TG in the secretory and endocytotic pathway. Second, we are trying to identify the determinants of the binding domain that acts as sensors, that promote or prevent the interaction with TG during its intracellular transit in post-ER compartments.

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REFERENCES

1. Van Herle, A. J., Vassart, G., and Dumont, J. E. (1979) New Engl. J. Med. 301, 307–314
2. Bjorkman, U., and Ekholm, R. (1980) in Biochemistry of Thyroid Hormone Formation and Secretion (Greger, M. A., ed) pp. 88–125, Raven Press, Ltd., New York
3. Arvan, P., Kim, P., Kuliawat, R., Prabakaran, D., Muresan, Z., Yoo, S. U., and Hashon, S. A. (1997) Thyroid 7, 89–105
4. Dumont, J. E., Vassart, G., and Refetof, R (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 6th Ed., pp. 1843–1879, McGraw-Hill, New York
5. Kim, P., Bele, D., and Arvan, P. (1992) J. Cell Biol. 118, 451–459
6. Kuznetso, G., Chen, L. B., and Nigam, S. K. (1994) J. Biol. Chem. 269, 22990–22994
7. Kim, P., and Arvan, P. (1995) J. Cell Biol. 128, 29–38
8. Muresan, Z., and Arvan, P. (1997) J. Biol. Chem. 272, 26095–26102
9. Kuznetso, G., Chen, L. B., and Nigam, S. K. (1997) J. Biol. Chem. 272, 3057–3063
10. Domu, R. W., Lamb, R. A., Rose, J. K., and Helenius, A. (1993) J. Virol. 1903, 545–562
11. Consiglio, E., Shifrin, S., Yavin, Z., Ambesi-Impombiato, F., Rall, J. E., Salvatore, G., and Kain, L. D. (1981) J. Biol. Chem. 256,10592–10599
12. Bastian, P., Papadrou, M. J., Blanck, O., Francinou, E., Thibault, V., and Miquelis, R. (1995) Endocrinology 136, 4204–4209
13. Ericson, L. E. (1981) Mol. Cell. Endocrinol. 13, 272, 13524–13533
14. Arvan, P., and Lee, J. (1991) J. Cell Biol. 112, 256–375
15. Edelhoch, H. (1985) in Thyroglobulin: The Prothyroid Hormone (Eggow, M. C., and Burrow, G. N., eds) pp. 1–12, Raven Press, New York
16. Zheng, C., Marino, M., Zhao, J., and McCluskey, E. T. (1988) Endocrinology 139, 1462–1465
17. Marino, M., Zheng, G., and McCluskey, R. T. (1990) J. Biol. Chem. 274, 20998–21004
18. Bastian, P. Simon, C., and Penel, C. (1980) Acta Endocrinol. 94, 4559–4563
19. Wiest, D. L., Bhandoola, A., Punt, J., Kreibich, G., McKean, D., and Singer, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 23340–23346
20. Miquelis, R. (1995) J. Biol. Chem. 272, 23340–23346
21. Wiest, D. L., Bhandoola, A., Punt, J., Kreibich, G., McKean, D., and Singer, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1884–1889
22. Bruneau, N., and Lombardo, D. (1995) J. Biol. Chem. 270, 13524–13533
23. Bruneau, N., Lombardo, D., and Bendayan, M. (1998) J. Cell Sci. 111, 2665–2679
24. Noiva, R., and Lennarz, W. J. (1992) J. Biol. Chem. 267, 3555–3556
25. Freedman, R., Hirst, T. R., and Tuite, M. P. (1994) Trends Biochem. Sci. 19, 331–336
26. Gilbert, H. P. (1997) J. Biol. Chem. 272, 29039–29042
27. Kroning, K., Kahne, T., Ittenson, A., Franke, A., and Ansorge, S. (1994) Scand. J. Immunol. 39, 346–350
28. Tager, M., Kroning, H., Thiel, U., and Ansorge, S. (1997) Exp. Hematol. 25, 601–607
29. Chen, K., Lin, Y., and Tetiuler, T. C. (1992) Blood 79, 2226–2228
30. Essex, D. W., Chen, K., and Swiatkowska, M. (1995) Blood 86, 2168–2173
31. Yosimori, T., Semba, T., Takemoto, H., Akagi, S., Yamamoto, A., and Tashiro, Y. (1990) J. Biol. Chem. 265, 15984–15990
32. Takada, K., Manchiklapudi, P., Noiva, R., Jauregui, J. O., Stockert, R. E., and Schilsky, M. J. (1995) J. Biol. Chem. 270, 24045–24046
33. Yoshimori, T., Semba, T., Takemoto, H., Akagi, S., Yamamoto, A., and Tashiro, Y. (1988) J. Biol. Chem. 263, 15984–15990
34. Mandel, R., Ryser, J. P., Ghani, F., and Peak, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 91, 4559–4566
35. Ryser, J. P., Levy, E., Mandel, R., and DiScheul, G. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4559–4563
36. Couet, J., deBernard, S., Loofsett, H., Saunier, B., Milgrom, E., and Mirahi,
pH-dependent PDI/Thyroglobulin Interactions in FRTL5 Cells

M. (1996) Biochemistry 35, 14800–14805
39. Krishna Rao, A. S. M., and Hausman, R. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2950–2954
40. Jiang, X. M., Fitzgerald, M., Grant, C. M., and Hogg, P. J. (1999) J. Biol. Chem. 274, 2416–2423
41. Berndorfer, U., Wilms, H., and Herzog, V. (1996) J. Clin. Endocrinol. Metab 81, 1918–1926
42. Delom, F., Lejeune, P. J., Vinet, L., Carayon, P., and Mallet, B. (1999) Biochem. Biophys. Res. Commun. 255, 438–443
43. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
44. Malthiéry, Y., and Lissitzky, S. (1987) Eur. J. Biochem. 165, 491–498
45. Parma, J., Christophe, D., Pehl, V., and Vassart, G. (1987) J. Mol. Biol. 196, 769–779
46. Molina, F., Bouanani, M., Pau, B., and Granier, C. (1996) Eur. J. Biochem. 240, 223–233
47. Nigam, S. K., Goldberg, A. L., Ho, S., Rohde, M. F., Bush, K. T., and Sherman, M. (1994) J. Biol. Chem. 269, 1744–1749
48. Munro, S., and Pelham, H. R. B. (1987) Cell 48, 899–907
49. Griffiths, G., Ericsson, M., Krijnsse-Locker, J. Nilsson, T. Goud, B., Soling, H.-D., Tung, B. L., Wong, S. H., and Hong, W. (1994) J. Cell Biol. 127, 1557–1574
50. Dorner, A. J., Wasley, L. C., Raney, P., Haugejorden, S., Green, M., and Kaufman, R. J. (1990) J. Biol. Chem. 265, 22029–22034
51. Hotchkiss, K. A., Chesterman, C. N., and Hogg, P. J. (1996) Biochemistry 35, 9761–9767
52. Stathakis, P. Fitzgerald, M., Matthias, L. J., Chesterman, C. N., and Hogg, P. J. (1997) J. Biol. Chem. 272, 20641–20645
53. Gilbert, H. F. (1998) Methods Enzymol. 90, 26–50
54. Anderson, R. G., and Patak, R. K. (1985) Cell 40, 635–643
55. Orci, L., Ravazzola, M., Amherdt, M., Perrelet, A., Powwell, S. K. Quinn, D. L., and Moore, H. P. (1987) Cell 51, 1039–1051
56. Demaurex, N., Furuya, W., D’Souza, S., Bonifacino, J. S., and Grinstein, S. (1998) J. Biol. Chem. 273, 2044–2051
57. Okhuma, S., and Poole, B. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3327–3331
58. Murphy, R. F., Powers, C., and Cantor, C. R. (1984) J. Cell Biol. 98, 1757–1762
59. Maxfield, F. R. (1989) Methods Enzymol. 173, 745–771
60. Thiele, C., Gerdes, H. H., and Huttner, W. B. (1997) Curr. Biol. 7, 496–500
61. Blanck, O., Perrin, C., Mziaut, H., Darbon, H., Mattei, M. G., and Miquelis, R. (1994) Genomics 21, 18–26
62. Blanck, O., Perrin, C., Mziaut, H., Darbon, H., Mattei, M. G., and Miquelis, R. (1998) Genomics 27, 561
63. Thibault, V., Blanck, O., Courageot, J., Pachetti, C., Perrin, C., De Mascarel, A., and Miquelis, R. (1993) Endocrinology 132, 468–476