For thyroid hormone synthesis, thyroid peroxidase (TPO) molecules must be transported from the endoplasmic reticulum via the Golgi complex to be delivered at the cell surface, in order to catalyze iodination of secreted thyroglobulin. Like other glycoproteins, TPO molecules in transit to the cell surface have the potential to acquire endoglycosidase H resistance as a consequence of Golgi-based modification of their N-linked carbohydrates; and measurement of the intracellular distribution of TPO has often relied on this assumption. To examine TPO surface distribution in thyrocyte cell lines, we prepared new antibodies against rat TPO. Antibody reactivity was first established upon expression of recombinant rTPO in 293 cells, which were heterogeneous for surface expression as determined by flow cytometry. By cell fractionation, surface rTPO fractionated distinctly from internal pools of TPO (that co-fractionate with calnexin), yet surface TPO molecules remained endo H sensitive. Although the FRTL5 (and PC Cl3) rat thyrocyte cell line also exhibits almost no endo H-resistant TPO, much of the endogenous rTPO is localized to the cell surface by immunofluorescence. Similar results were obtained by fractionation of FRTL5 cell membranes on sucrose gradients. We conclude that in FRTL5 cells, a large fraction of rTPO is delivered to the plasma membrane yet does not acquire Golgi-type processing of its N-glycans. Rat and mouse thyroid tissue TPO also shows little or no endo H resistance, although cell fractionation still needs to be optimized for these tissues.

In the biosynthesis of thyroid hormones, iodination of thyroglobulin occurs primarily at the apical surface of thyrocytes delimiting the thyroid follicle lumen. The iodination reaction is catalyzed by thyroid peroxidase (TPO) (1), a type 1 membrane protein whose enzymatic activity is extracellularly disposed at the surfaces of microvilli of the apical plasmalemma (2). Part of the apical extracellular selectivity of iodination may derive from the localization of NADPH oxidases that provide H$_2$O$_2$ (3), an essential substrate for TPO (4), in conjunction with active degradation of cytoplasmic H$_2$O$_2$ (5). TPO has been identified as the major antigen to which thyroid anti-microsomal antibody is directed (6). Patients’ sera immunolocalize the thyroid microsomal antigen at the apical cell surface of thyrocytes (7) while acute TSH stimulation further augments cell surface TPO enzymatic
activity/immunoreactivity (8-13). Nevertheless, like other plasma membrane proteins, TPO is first synthesized within the endoplasmic reticulum (ER) where it can be readily detected (14-16). After folding to the native state within the ER (17; 18), intracellular transport of TPO to the cell surface occurs via the Golgi complex—a compartment typically associated with N-glycan processing of many cell surface glycoproteins (19).

One potential cause of congenital goitrous hypothyroidism includes expression of mutants of TPO that fail to exit from the endoplasmic reticulum (20-22), and this might occur also for a subpopulation of wild-type TPO, especially because of mRNA splicing that may result in production of trafficking-defective isoforms (23). In thyroid follicles, the cell surface fraction of TPO is especially difficult to study because the apical plasmalemma is experimentally inaccessible. In thyrocyte primary cultures, the fraction of total cellular TPO protein residing at the plasma membrane is limited (24). While this might be caused by splice variants, there may be additional confounding variables such as those introduced by protease-based cell dissociation procedures (that may cleave the TPO ectodomain) as well as by TPO sequestration within intracellular lumina that appear during thyrocyte primary culture (25).

Two tools have been used extensively in the hope of circumventing some of the technical challenges to the study of TPO trafficking. First, as a surrogate for thyrocytes, recombinant TPO has been studied in nonthyroid cells where experimental access to the plasma membrane is straightforward; and from such studies it has also been suggested that the fraction of TPO molecules at the cell surface may be very limited (26). Second, sensitivity of TPO N-glycans to digestion with endoglycosidase H might be used to provide an estimate of the fraction of TPO molecules residing within or beyond the endoplasmic reticulum (18). However, as straightforward as these methods are to execute, we previously reported that cell type-dependent differences in TPO surface expression may limit the utility of recombinant TPO for physiological studies of intracellular distribution, and further, we have raised the question of whether TPO molecules exiting the ER necessarily acquire endoglycosidase H resistance (27). Indeed, it is a well-recognized phenomenon that the glycans on some exportable proteins fail to acquire endo H-resistance upon trafficking through the Golgi en route to the plasma membrane—perhaps due to physical inaccessibility of high mannose oligosaccharides in the tertiary or quaternary structure (28). Numerous studies have documented the presence of certain proteins that remain endo H-sensitive at the plasma membrane (29) or in the secretion from cells [for which RNase B is the prototype (30)]. It is also clear that the same glycoprotein may achieve complex glycosylation in one cell type yet not receive complex glycosylation in another cell type (31).

For all of these reasons we have been interested to develop a system with which to pursue the distribution of endogenous TPO at the cell surface, using thyrocyte cell lines that express thyroid differentiated function (32). Probably the best studied thyrocyte cell line is the rat-derived, FRTL5 cell model. In these cells, TPO mRNA levels have been reported in numerous studies but no examination of the endogenously expressed TPO protein has yet been performed. Indeed, heretofore we were unable to identify polyclonal antisera against hTPO that could unequivocally identify endogenous rTPO protein (27). Therefore, we set out to prepare an antiserum
against rTPO, in order to begin to characterize behavior of the protein in transfected cells, in thyrocyte cell lines, and in thyroid tissue.

EXPERIMENTAL PROCEDURES

Materials The full-length rTPO cDNA was the kind gift of Dr. R. Magnusson (Mt. Sinai Medical Center, New York NY). The cDNA was subcloned into pCDNA3 (Invitrogen) in which hTPO expression was driven by the immediate early CMV promoter. Recombinant endoglycosidase H was from New England Biolabs and was used (1,000 units per 50 ul digest) according to the manufacturer’s instructions. Enhanced chemiluminescence substrate for Western blotting was from Pierce.

Antibodies and Blotting Reagents An amphipathic profile of the rTPO amino acid sequence showed four peak regions within the protein that would predict high antigenicity. We selected a peptide encoding residues 475 - 490 of rTPO (TAAFRFGHATVHPLVR) for peptide synthesis. [A sequence with a high degree of similarity (but non-identity) to this sequence can be found in 5 distinct reported isoforms of human TPO (Accession numbers NP_000538, NP_783650, NP_783651, NP_783652, NP_783653)]. The synthesized peptide included an N-terminal CKK linker sequence. The N-terminal cysteine allowed for directed coupling of the peptide to maleimide-activated keyhole limpet hemocyanin (KLH) while the linker lysines provided improved peptide solubility in aqueous solution. New Zealand White rabbit immunization of the KLH-conjugated rTPO peptide was as per standard protocol (Covance Immunochemicals, Denver PA). Specific immunoglobulins were enriched from crude antisera in two ways. First, an IgG-enriched fraction was prepared by passing the anti-TPO serum over a protein A-Sepharose column and eluting in a low pH glycine buffer followed by pH neutralization (this resulted in significant dilution of the original antiserum). Second, the IgG-enriched fraction was incubated overnight at 4°C with intact, formaldehyde-fixed 293 cells, in order to desorb immunoglobulins that react nonspecifically with the extracellular surface of 293 cells. In different experiments, Western blotting was performed with IgG-enriched fraction that had either been desorbed or not; the final IgG-enriched fraction was diluted at 1:500 or 1:1000 in different experiments.

A rabbit antiserum against gamma glutamyl transpeptidase (33) was graciously provided by Prof. J. David Castle (U. Virginia, Charlottesville) and was used for immunoblotting at a dilution of 1:4,000. A rabbit antiserum against aminopeptidase N was provided by Dr. Hans Sjostrom (U. Copenhagen, Denmark) at a dilution of 1:4,000. A rabbit antiserum against calnexin (diluted 1:4000) was provided by Dr. Paul Kim (Geisinger Medical Ctr. Danville, PA). For secondary antibodies, goat anti-rabbit (horseradish peroxidase conjugate diluted 1:5,000) was from Jackson Immunochemicals.

Cell Culture FRTL5 and PC Cl3 cells were grown in Coon’s medium plus 5% calf serum, 10 uU/ml TSH, 1 ug/ml insulin, 5ug/ml transferrin, and 10 nM hydrocortisone. 293 cells were grown in DMEM plus 10% fetal bovine serum on regular tissue culture plasticware. To passage these cells, they were dissociated from plates using enzyme-free, Hanks’-based Cell Dissociation Buffer (Invitrogen, catalog #13150-016). The cells were incubated at 37°C in a 5% CO2 incubator until 50-80% confluent. 293 cells were transfected with lipofectamine. After overnight incubation with the cells, the medium was replaced with fresh complete growth medium. For transient
transfection, assays were performed after 48 h. For stable transfection, the cells were passaged to large Petri dishes and incubated in complete medium containing 0.8 mg/ml G418. Individual clones were isolated, expanded, and maintained in medium containing 0.4 mg/ml G418. Antibiotics (streptomycin-penicillin from Gibco/BRL) were added to all cell culture media.

**Immunofluorescence** FRTL5 cells were grown on uncoated glass coverslips (Bellco) and fixed with 3.7% formaldehyde. After washing in PBS, cells were permeablized with 0.1% Triton X-100 in a blocking solution containing 3% BSA in PBS. Anti-TPO enriched IgGs (that had been pre-diluted in preparation) were further diluted 1:200 before incubation with the fixed, permeabilized cells. In addition the cells were incubated with a mAb anti-protein disulfide isomerase (Stressgen) at a dilution of 1:300. After extensive washing, the cells were incubated with donkey anti-rabbit Cy2 conjugate diluted 1:500, and with donkey anti-mouse Cy3-conjugate at 1:400. The cells were washed finally before mounting and visualization with a Nikon ECLIPSE E400 microscope equipped with epi-fluorescence optics and a digital camera. Negative controls omitting primary antibodies exhibited a black background (ie, no fluorescence) at the exposure times employed for specific immunofluorescence.

**Sucrose Velocity Gradient Fractionation** In all cell fractionation experiments described in this study, cells were initially homogenized in 0.3 M sucrose, 0.2 mM MgSO4, 10 mM Hepes pH 7.4. In each case 0.5 mL of homogenate was spun at 1,500 x g x 2min to sediment nuclei and the postnuclear supernate was used for further analysis by fractionation. Differential centrifugation was performed as described in the text. For improved resolution of plasma membrane vesicles from rough endoplasmic reticulum, samples were analyzed by sucrose velocity gradients. Each 2.2 ml gradient was comprised as follows: 0.950 ml of 38% sucrose, 10 mM Hepes, pH 7.4; 0.475 ml of 30% sucrose, 10 mM Hepes, pH 7.4; 0.475 ml of 25% sucrose, 10 mM Hepes, pH 7.4; and 0.300 ml of sample loaded at the top. Samples were then loaded into a swinging bucket RP55S rotor and spun at 167,000 x g for 120 min in an RC-M120EX (Sorvall). Equal volume fractions were collected manually beginning from the top of each tube and processed as described in the text.

**RESULTS**

**Expression of rTPO in 293 cells** As described in Experimental Procedures, we prepared a new rabbit antiserum directed against residues 475 - 490 (TAARFGRGATVHLPR) of rTPO. To test the reactivity of immunoglobulins enriched from this serum, we compared untransfected 293 cells (a negative control) to those transiently transfected with the rTPO cDNA (a positive control). By Western blotting, a number of nonspecific bands were evident, including a band at ~105 kDa. Upon transient expression of rTPO, in addition to nonspecific background bands was a strongly immunolabeled ~110 kDa band (Fig. 1, upper left). Upon mock-digestion or digestion with endo H (upper middle panel), most if not all ~110 kDa rTPO converted from the undigested position ("TPO.u ") to the digested, endo H-sensitive band ("TPO.s"). Similarly, rTPO was fully digested with PNGase F (Fig. 1, upper right). These data indicate that a) our anti-peptide antiserum specifically recognizes rTPO, b) all rTPO molecules expressed in 293 cells acquire N-glycosylation, and c) most rTPO in
293 cells is endo H sensitive. Since we wished to further explore these features, it was a matter of convenience to prepare stable rTPO-expressing clones of 293 cells. More than 50 such G418-resistant clones were screened by immunoblotting, with a broad range of rTPO expression levels. An example of such screening is shown in Fig. 1 (lower panels). We ultimately selected a few strongly-expressing clones for further study (each of which exhibited identical rTPO behavior), concentrating on a clone designated 293-22.

**Clone 293-22 versus rat thyrocytes**

When normalized for total homogenate protein, clone 293-22 expressed more Western blottable rTPO than PC Cl3 or FRTL5 rat thyrocyte cell lines that express endogenous rTPO (Fig. 2). It was difficult to state the relative rTPO expression levels in 293-22 cells versus authentic rodent thyroid tissue because of "contamination" of thyroid tissue by follicular thyroglobulin, which is the most abundant thyroid tissue protein that influences normalization of SDS-PAGE samples loaded on a per-protein basis. Knockout mice lacking prohormone convertase 1 (expected to be defective for processing of pro-thyrotropin releasing hormone (34)) yielded thyroid tissue (that is likely to contain a decreased content of follicular thyroglobulin) with reproducibly less rTPO protein than thyroids from wild-type control mice (lanes 6-9). The essential point is, regardless of the source of expression, the overwhelming majority of rTPO protein was endo H sensitive. Only in the strongest-expressing situation (293-22 cells) was a distinct endo H resistant TPO band clearly discernible, although this band accounted for only a few percent of total cellular TPO [as has been previously described for CHO cells expressing hTPO (26)].

We and others have previously reported that recombinant TPO expression, even in clones derived from single cells, results in two cell subpopulations each of which maintain comparable total TPO protein expression but only one of which maintains surface TPO protein expression (27). Therefore, we bathed live 293-22 cells expressing rTPO in medium containing anti-rTPO and a fluorescent-conjugated anti-rabbit secondary antibody, and used flow cytometry and fluorescence-activated cell sorting (FACS) to segregate the cells into FACS-negative and FACS-positive subpopulations (Fig. 3, upper panel). Both FACS-negative and FACS-positive subpopulations were further expanded in cell culture and the FACS positive subpopulation put through a second iterative round of FACS to segregate and maximally enrich surface-positive cells. Samples of these subpopulations were examined thereafter by immunofluorescence microscopy (of the nonpermeabilized, live cells), visually confirming enrichment of surface-expression in FACS-positive 293 cells (Fig. 3, lower panels). The TPO expressed in these subpopulations was then analyzed for endo H sensitivity. As shown in Fig. 4, when normalized for equal protein, neither first- nor second-round FACS-positive cells ("FACS1", "FACS2") showed enrichment of endo H-resistant TPO over that present in the original 293-22 clone. The same was true for cells lysated immediately after the FACS procedure as well as for cells put into culture after FACS. Curiously, the suggestion of a very slightly increased level of endo H-resistant TPO actually appeared in FACS-negative cells (Fig. 4).

To further explore this point, we exploited differential centrifugation, which has been used as an effective and rapid means to enrich a plasma membrane fraction away from the endoplasmic reticulum of 293 cells. A postnuclear supernate (0.5 mL) was sedimented at 14,000 x g for 4 min.
to generate S (supernate) and P (pellet) fractions. Gamma glutamyl transpeptidase (GGT), a plasma membrane marker of 293 cells (35), was located primarily in the S fraction, while the ER marker calnexin (CNX) was located primarily in the P fraction (Fig. 5A). When comparing FACS-negative and FACS-positive cells, the latter had an obvious increase in Western blottable TPO in the S fraction (Fig. 5A), consistent with an increased fraction of TPO molecules at the surface of these cells. However, while GGT did not significantly contaminate the P fraction, some calnexin was in fact recovered in the S fraction, indicating some degree of contamination of this fraction by ER membranes (Fig. 5A). We therefore compared the behavior of TPO in the S and P fractions by loading these membranes atop sucrose gradients that provide improved separation of plasma membranes (upper region of the gradient) from the ER (lower region). Indeed, sucrose gradient analysis of TPO-containing membranes from the S fraction yielded a distinct sucrose gradient distribution from that of the P fraction (Fig. 6), supporting that they are in different compartments. Specifically, the former distributed in the upper region of the gradient while latter distributed near the bottom, co-sedimenting with calnexin (CNX).

As a further measure of enrichment for plasma membrane TPO, we biotinylated the cell surface of FACS-positive cells with the non-membrane-permeant reagent, sulfo-NHS-biotin. We prepared the S fraction from the surface-biotinylated cells and then treated this fraction by precipitation with avidin-agarose. When the undigested ~110 kDa TPO molecules in this fraction were further analyzed ("TPO.u", Fig. 5B), the preponderance of these surface molecules converted to the TPO.s position after endo H digestion, indicating endo H sensitivity similar to that observed in the P fraction or in a total rat thyroid tissue homogenate (Fig. 5B).

**Endogenous Expression of rTPO in FRTL5 cells** As noted, our rTPO antiserum immunoreacted specifically with rodent thyroid tissue (Figs. 2, 6B) as well as PC Cl3 and FRTL5 thyrocyte cell lines (Fig. 2). These cell lines, like most adherent cells in culture, are passaged routinely by trypsinization, during which the cells remain viable and after which they are expanded by growth on tissue culture plates. Cleavability by trypsin added to nonpermeabilized living cells is a well recognized and sensitive assay of cell surface distribution for membrane proteins with a protease-accessible ectodomain (36; 37), whereas trypsinization of live cells cannot cleave proteins residing within the ER. We therefore exploited trypsinization as a means to examine the surface distribution of TPO in FRTL5 cells. For these studies, we simultaneously examined another plasma membrane marker protein that might serve as a valuable basis for comparison. In addition to its expression in 293 cells (Fig. 5A), gamma-glutamyl transpeptidase (GGT) is a protein that resides on the cell surface in a number of exocrine cell types including kidney cells and the rat parotid gland (38) and has been found in thyroid carcinomas (39). When compared to parotid tissue as a positive control, GGT is also clearly expressed endogenously in normal rat thyroid (Fig. 7, left panel) and in FRTL5 cells (see below). FRTL5 cells were dissociated from tissue culture plasticware either by routine trypsinization or by cell scraping, which eliminates the use of exogenous proteases. When analyzed by immunoblotting normalized to equal total cellular protein, after trypsin exposure of FRTL5 cells there was a 90% reduction in detectable ~110 kDa rTPO (Fig. 7, bottom right panel). This result has been repeated and confirmed several times.
The fact that a large fraction of GGT (an inherently more trypsin-resistant cell surface polypeptide) remained detectable from the trypsinized cells strongly suggests that the results with TPO do not reflect nonspecific cleavage occurring artifactually after sample preparation for SDS-PAGE, but rather, that TPO is highly trypsin-sensitive and a major fraction is surface-accessible in FRTL5 cells.

Next, we examined the distribution of rTPO in FRTL5 by double labeling immunofluorescence. In contrast to protein disulfide isomerase (red), which exhibited primarily an ER-staining pattern, rTPO (green) exhibited bright staining at the cell surface (Fig. 8). To confirm these findings, a post-nuclear supernate of FRTL5 cells was analyzed by sucrose gradient centrifugation. The plasma membrane marker GGT was recovered primarily in the three upper fractions of the gradient (Fig. 9). Aminopeptidase N (APN), another known thyroid plasma membrane marker (40), was similarly enriched in the upper region of the gradient (Fig. 9). While the ER marker calnexin was distributed to the bottom region of the gradient, TPO exhibited a distribution similar to that of APN or GGT (Fig. 9). Taken together, the data in Figs. 7-9 suggest that most rTPO resides at the surface of FRTL5 cells.

**Endogenous Expression of rTPO in Rat Thyroid Tissue**  In order to further study the distribution of TPO expressed endogenously in rat thyroid tissue, preliminary cell fractionation studies have been undertaken. Obviously, less well controlled homogenization and the potential for overloading the protein capacity of sucrose gradients are a greater risk in the analysis of real thyroid tissue compared to those from FRTL5 cells. Despite the overlap, distributions of the markers remained distinct, and the pattern for TPO from rat thyroid tissue looked much more like that of APN than that of CNX. Curiously, a band of ~90 kDa (in fractions 2 and 3) was detected in the sucrose gradients derived from rat thyroid tissue, and we do not yet know if this is an authentic TPO splice form (41; 42). Nevertheless, these preliminary experiments do support the conclusion that TPO is well expressed at the plasma membrane of thyrocytes from real thyroid follicles.

**DISCUSSION**

TPO is a protein that must undergo intracellular transport from the ER to the plasmalemma in order to execute its physiological function. The intracellular distribution of TPO protein along the secretory pathway has long been a confusing issue. In thyroid follicles, it is especially difficult to estimate the (apical) surface fraction of TPO because of its experimental inaccessibility. In primary porcine thyrocytes after 7 days in culture, ~30% of endogenously expressed TPO could be biotinylated at the cell surface (40), and a similar number has been reported for recombinant hTPO (41). Another study proposed that only 2% of cellular TPO may reside at the plasmalemma (26), but there are reasons to wonder if all of these studies might represent great underestimates of the TPO surface distribution [both because of intracellular lumina that may sequester plasmalemmal proteins during thyrocyte primary culture (25) and because of low biotinylation efficiency of surface TPO (27)]. Alternatively, some investigators have examined
acquisition of endo H resistance to estimate the fraction of cellular TPO molecules that have proceeded beyond the ER through the secretory pathway, and this fraction is very low in real thyroid tissue (Figs. 2, 5B). Nevertheless in thyrocytes, TPO glycans may not be efficiently modified (43) and the extent of post-translational processing may be different in nonthyroid cell types expressing recombinant TPO (27).

For these reasons, it is of interest to examine thyrocyte cell lines in which thyroid differentiated gene products (including TPO) are endogenously expressed. Unfortunately, rat-derived FRTL5 and PC Cl3 lines, which are most commonly used, both express rTPO that does not appreciably cross-react with anti-hTPO antibodies or IgGs of patients with Graves’ disease. In this study we prepared new rabbit polyclonal antibodies against a synthetic peptide which yields suitable immunoreactivity against rTPO (Fig. 1). Like many anti-peptide antibodies, the new anti-rTPO antibodies are not suitable for recognition of the native antigen (such as in immunoprecipitation) but work well for Western blotting after SDS-PAGE, or immunofluorescence after formaldehyde fixation of FRTL5 cells.

In clones of 293 cells stably expressing recombinant TPO, similar to previous reports of CHO cells (27), there is heterogeneity of surface expression amongst the cell population (Fig. 3). Nevertheless, even when only surface-positive cells are considered, there is still no appreciable enrichment of endo H-resistant TPO (Fig. 4). From surface-positive cells we can use differential centrifugation to enrich for surface TPO molecules (Fig. 5A); and further enrich by biotinylating the cell surface TPO before cell fractionation and then using avidin-agarose to isolate surface TPO molecules selectively from the plasma membrane-enriched fraction (Fig 5B). We are confident that our plasma membrane-enriched fractions of FRTL5 cells or 293 cells have little contamination by ER membranes because calnexin (the ER membrane protein marker) is efficiently separated from plasma membrane protein markers by sucrose velocity gradient centrifugation (Figs. 6, 9). Despite the lack of contamination by ER, the ~110 kD surface TPO molecules remain endo H sensitive in 293 cells (Fig. 5B) and FRTL5 cells (Fig. 1). Thus, lack of Golgi-glycan processing of the ~110 kDa TPO cannot be used to predict protein residency in pre-Golgi compartments.

It has been recently shown that recombinant hTPO may be subject to endoproteolysis during its secretory trafficking in heterologous cells (18). We do not yet know the extent to which this occurs in thyrocytes. However, we find that when passaging FRTL5 thyrocytes in culture, detectability of cellular TPO is eliminated, suggesting high sensitivity and surface accessibility to cleavage by exogenous trypsin (Fig. 7). By immunofluorescence in FRTL5 cells, endogenous TPO exhibits a strong signal from the plasmalemma which is dramatically different from that of the ER marker, protein disulfide isomerase (Fig. 8). In conjunction with the results from sucrose velocity gradient fractionation (Fig. 9), these results, taken together, indicate that a large fraction of cellular TPO in the rat thyrocyte cell line, FRTL5, is distributed at the plasma membrane.

Finally, we have begun to turn attention to the intracellular distribution of TPO in real thyroid tissue. Our homogenization and fractionation scheme for FRTL5 cells has not been as successful when using whole thyroid tissue as a starting material. Thus, we cannot exclude a significant steady state distribution of rTPO in the ER of the rat thyroid gland. Moreover, by immunoblotting we can also identify a lower molecular weight
band that might possibly be a TPO-related isoform or proteolytic fragment (Fig. 10), but this has not been confirmed. For the ~110 kDa TPO band, our impression remains that its distribution is significantly different from that of the ER marker calnexin, and more similar to that of the plasma membrane marker aminopeptidase N (Fig. 10).

More work is still needed to evaluate the claim that protein folding difficulties normally limit the distribution of wild-type TPO to the follicular cell surface in the thyroid tissue of vertebrate organisms (17; 26; 44).

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Figure 1. TPO expression in 293 cells. The upper panels show immunoblotting with anti-rTPO of 293 cells mock-transfected with pCDNA3 empty vector (""") or transfected to express the rTPO cDNA ("+"). In several immunoblots, a nonspecific band at 105 kD can be observed in mock-transfected cells. A slower migrating, specific immunoreactive ~110 kDa band appears in cells transfected to express rTPO. The ~110 kDa band represents the mobility of undigested rTPO ("TPO.u") but this band is converted to a faster migrating band ("TPO.s") indicating sensitivity to digestion with endoglycosidase H ("Endo H") or PNGase F. The lower panels show individually selected G418-resistant clones of 293 cells, indicating a range of rTPO expression amongst clones. The clone employed in most of the experiments in this study, called 293-22, exhibits among the highest rTPO expression levels, yet a distribution of rTPO that is comparable to the other clones displayed here.

Figure 2. TPO expression in cells and tissues. Eighty micrograms per lane of cell or tissue lysate protein for each sample was taken for analysis of rTPO protein expression by SDS-PAGE and immunoblotting. Lanes 6 and 7 are derived from thyroid tissue of C57BL/6j mice lacking prorhormone convertase 1 whereas lanes 8 and 9 are from wild-type control mice (45).

Figure 3. 293-22 cells (expressing rTPO, growing in continuous culture) were exposed to anti-rTPO antibodies and a FITC-conjugated anti-rabbit secondary antibody in PBS. Upper panel: flow cytometry analysis of the cells. Shortly after FACS, the cells were returned to tissue culture plasticware, and low power microscopic images by both phase-contrast (middle panels) and surface immunofluorescence (lower panels) were captured on an inverted tissue culture microscope.

Figure 4. Clone 293-22 cells, or cells derived from 293-22 cells that had been segregated a single time (FACS1) or twice sequentially (FACS2) for positive TPO-surface immunofluorescence were analyzed by SDS-PAGE and immunoblotting, in comparison to rTPO-expressing 293 cells that were selected for an absence of surface immunofluorescence (FACS—). Each cell lysate was either mock digested ("—") or digested ("+") with endo H before SDS-PAGE.

Figure 5. Differential centrifugation of clone 293-22 cells expressing rTPO. A) After lysing cells in a ball-bearing homogenizer and preparing a postnuclear supernate, the samples were sedimented at 14,000 x g x 4 min to generate S (supernate) and P (pellet) fractions. The fractions were immunoblotted for gamma glutamyl transpeptidase ("GGT", the heavy and light chains are apparent but the unprocessed form is not shown), calnexin ("CNX"), and rTPO. B) The surface of 293-22 cells was biotinylated with sulfo-NHS-biotin and the reaction quenched before cell lysis. Differential centrifugation of the postnuclear supernate was performed as in A) and the supernate then precipitated with avidin-agarose to further enrich for cell surface proteins. The avidin-agarose pellet was then either mock-digested ("—") or digested ("+") with endo H before analysis by SDS-PAGE and immunoblotting. The position of the ~110
kDa rTPO band is shown in comparison to the rTPO band expressed in the pellet of the differential centrifugation and the undigested (TPO.u) or digested (TPO.s) TPO expressed in whole rat thyroid tissue.

Figure 6. Sucrose gradient fractionation of membranes containing rTPO. Differential centrifugation of a postnuclear supernate from clone 293-22 was performed as in Fig. 5. The S (supernate) and P (pellet) fractions were then loaded atop sucrose velocity gradients and analyzed as described in Experimental Procedures. After ethanol precipitation of each fraction collected from the top (left), the samples were analyzed by SDS-PAGE. Immunoblotting of the ~110 kD rTPO and ~90kDa calnexin (CNX) bands are shown.

Figure 7. Sensitivity of thyroid plasma membrane proteins to exogenous trypsin. Panel at left: samples of equal protein content from normal rat thyroid tissue were run side-by-side with rat parotid tissue by SDS-PAGE followed by immunoblotting for GGT (both specific unprocessed and processed forms are shown); demonstrating thyrocyte expression of GGT in comparison to the positive control. Panels at right: Live FRTL5 cells were either trypsinized (+) or scraped (−) from tissue culture plastic dishes and then analyzed by SDS-PAGE and immunoblotting, normalized to equal cell protein. After trypsinization, the rTPO band was decreased by >90%.

Figure 8. Indirect immunofluorescence of rTPO (green) and protein disulfide isomerase (red) in FRTL5 cells. The antibodies and method is described in Experimental Procedures.

Figure 9. Sucrose gradient fractionation of FRTL5 cell membranes. FRTL5 cells were homogenized and a postnuclear supernate, generated as described in Experimental Procedures, was loaded atop sucrose gradients. After ethanol precipitation of each fraction collected from the top (left side of Figure), the samples were analyzed by SDS-PAGE. Immunoblotting of the ~160 kDa aminopeptidase N (APN), the ~110 kD rTPO, the ~90kDa calnexin (CNX), and the two specific GGT bands are shown.

Figure 10. Sucrose gradient fractionation of rat thyroid cell membranes. Thyroids were homogenized and a postnuclear supernate, generated as described in Experimental Procedures, was loaded atop sucrose gradients. After ethanol precipitation of each fraction collected from the top (left side of Figure), the samples were analyzed by SDS-PAGE. Immunoblotting of the ~110 kD rTPO, the ~160 kDa aminopeptidase N (APN), and the ~90kDa calnexin (CNX) bands are shown.
Transient Expression in 293 Cells

Transfection: +  -  
Endo H: -  +  
PNGase F: -  +

293-TPO Clones: 1 2 13 14 15 18 21 22 26 34

Kuliat et al., Fig. 1
Cells: 293  293-22  PC Cl3  mThyroid  mThyroid  FRTL5
Endo H:  -  -  +  -  +  -  +  -  +  -  +

Kuliawat et al., Fig. 2
Kuiliawat et al., Fig. 3
Kulawat et al., Fig. 4
Kuliawat et al., Fig. 5