Cell-type dependent differences in thyroid peroxidase cell surface expression

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Running Title: Cell-type dependent TPO trafficking

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Recently it has been suggested that only ~2% of human thyroid peroxidase (hTPO$_{933}$) reaches the surface of stably transfected CHO cells — most being degraded intracellularly — and this might be representative of TPO behavior in thyrocytes [Fayadat et al. (2000) *J. Biol. Chem.* **275**, 15948-15954]. In agreement, in stably transfected MDCK clones, nonpermeabilized cells exhibit wild-type hTPO$_{933}$ immunofluorescence (apically) on <10% of that found in permeabilized cells, where an endoplasmic reticulum (ER) pattern is observed. Further, a C-terminally truncated, membrane-anchorless hTPO$_{848}$ is also retained in the ER of stably transfected MDCK cells. However, by contrast, in CHO cells after transient transfection, hTPO$_{933}$ immunofluorescence is detected equally well in nonpermeabilized and permeabilized cells, indicating that a large portion of hTPO$_{933}$ is present at the cell surface; furthermore, hTPO$_{848}$ is efficiently secreted. Further, using an antiserum not cross-reacting with rat TPO, we find by immunofluorescence that in stable clones of PC Cl3 (rat) thyrocytes, considerably more (~50%) of the cells exhibit hTPO$_{933}$ at the cell surface. However, cell surface biotinylation and endoglycosidase H digestion assays appear to underrepresent the extent of hTPO$_{933}$ transport, presumably because protein folding limits both Golgi carbohydrate modification and accessibility of lysines in the extracellular domain. We conclude that cell-type specific factors may facilitate stable expression of TPO at the cell surface of thyrocytes.
INTRODUCTION

In the past decade, cell-type dependent differences in membrane and secretory protein trafficking are increasingly recognized. Certainly, the rate and efficiency of protein folding and export from the endoplasmic reticulum (ER) varies between cell types (1). Further, in the distal secretory pathway, the expression of cDNAs encoding regulated secretory proteins has been found to result in storage in secretory granules in some regulated secretory cell types but not others (2-5). Also, cell surface polarity signals can be differentially interpreted by different polarized cell types (6-10). The identification of cell-type-dependent differences in protein sorting and trafficking represents a crucial first step in elucidating the underlying molecular mechanisms that account for such differences (11).

Thyroid peroxidase (TPO) is a key enzyme responsible for iodination of thyroglobulin during the synthesis of thyroid hormones. Electron microscope autoradiography studies have established that iodination activity is localized primarily at the (extracellular aspect of the) apical surface of thyrocytes delimiting the thyroid follicle lumen; suggesting that under physiological circumstances, TPO is primarily an apical plasma membrane enzyme (12). TPO is neither the sole gene product responsible for the diaminobenzidine oxidation reaction (a technique that has been used to detect peroxidase activity cytochemically) nor the sole protein immunoreacting with thyroid autoimmune antisera [used to immunolocalize the "anti-microsomal antigen" (13)]. Nevertheless, TPO cytochemical activity in thyrocytes is associated with apical membrane vesicles and the outer surfaces of apical microvilli (14), while additional reaction product can be detected in the thyrocyte ER and Golgi complex. Moreover using patients' antisera, the thyroid microsomal antigen is immunolocalized to the apical surface of thyrocytes (15), while additional immunoreaction is found in the cytoplasm (16; 17). Acute TSH stimulation of the thyroid gland causes additional TPO enzymatic activity and immunoreactivity at the apical cell surface (18-23). These results all indicate that TPO travels via the secretory pathway to the plasma membrane.

In spite of this, lingering questions persist about the trafficking of TPO to the cell surface in the thyroid and in various cell culture systems (24). In primary porcine thyrocytes after 7 days in culture, only ~30% of endogenously expressed TPO could be chemically modified at the cell surface with a non-permeant biotinylation reagent (25). Further, it has recently been reported that only ~2% of recombinant wild-type hTPO (full-length hTPO933) reaches the surface of a stably transfected CHO cell clone, while most hTPO933 is retained in the ER without intracellular transport (26; 27); and this fraction at the cell surface is only negligibly increased by enhanced heme availability (28). From this it has been argued that impaired intracellular traffic and massive...
degradation of recombinant hTPO\textsubscript{933} (29) might be representative of the situation found in thyrocytes.

In this report, we have compared the surface expression of recombinant hTPO in three very different cell culture models. Our data provide strong evidence to suggest that delivery of hTPO to the cell surface varies widely depending upon the system in which it is studied. We find that clonal MDCK cells fail to efficiently export wild-type hTPO\textsubscript{933} to the plasma membrane, and fail to secrete a truncated hTPO\textsubscript{848} luminal domain. However, we find that both hTPO\textsubscript{933} and hTPO\textsubscript{848} are exported quite efficiently in transiently transfected CHO cells in spite of the fact that after clonal CHO cell selection and expansion, only little hTPO\textsubscript{933} is found at the cell surface (26). More importantly, in stably transfected clones of PC Cl3 (rat thyrocyte) cells, we also find a significantly higher fraction of hTPO\textsubscript{933} distributed on the plasma membrane. These results suggest that cell-type specific factors may allow for stable surface expression of hTPO in thyrocytes.
MATERIALS AND METHODS

Materials

A rabbit antiserum (serum L-0666) against hTPO generously provided by Dr. A. Taurog (U.T. Southwestern, Dallas TX) was initially prepared by Dr. Paul Banga (King's College, London UK) against the whole purified molecule. The full-length hTPO933 and C-terminally truncated hTPO848 cDNAs were the kind gifts of Drs. R. Magnusson (Mt. Sinai Medical Center, New York NY) and B. Rapoport, Cedars-Sinai Medical Center, Los Angeles, CA). Both cDNAs were subcloned into pCDNA3 (InVitrogen) in which hTPO expression was driven by the immediate early CMV promoter. Sulfo-NHS-biotin (used at 0.6 mg/ml) was from Pierce (Rockford, IL). Avidin-agarose was from Roche Biochemicals. For secondary antibodies, goat anti-rabbit horseradish peroxidase conjugate and goat anti-rabbit Cy3 conjugate were from Jackson Immunochemicals. For immunoprecipitation, the secondary precipitant was Zysorbin (Zymed, So. San Francisco CA). The enhanced chemiluminescence substrate for blotting was from Amersham and the [35S]-amino acid mixture (Expre35S35S) was from NEN. Recombinant endoglycosidase H was from New England Biolabs and was used according to the manufacturer's instructions.

Cell Culture

MDCK strain II and CHO cells were grown in DMEM plus 10% fetal bovine serum. PC Cl3 cells were obtained from Dr. James Fagin (U. of Cincinnati, OH) and 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. Antibiotics (streptomycin-penicillin from Gibco/BRL) were added to all cell culture media.

Transfection

In 35-mm tissue culture dishes, 2 x10^5 MDCK, CHO or PC Cl3 cells were seeded in complete growth medium. The cells were incubated at 37°C in a 5% CO2 incubator until 50-80% confluent. For each transfection, 1-2 ug of plasmid DNA was diluted into 100ul Opti-MEM (GIBCO/BRL). Separately, 15 ul lipofectamine was diluted to 100ul in the serum-free medium. The two solutions were gently mixed and incubated for 30 min at room temperature, before diluting to 1 ml in serum-free medium. Each dish of cells was rinsed twice with the same medium before the lipofectamine/DNA mix was added. After overnight incubation with the cells, the medium was replaced with fresh complete growth medium. For transient transfection of CHO cells, assays were performed after 48 h. For stable transfection of MDCK and PC Cl3 cells, after 48 h, 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 selection medium for 3-6 months prior to experiments. Of note in one instance (Fig. 6B), 16 h before the experiment, cell culture medium was modified to include 10 mM sodium butyrate to enhance expression from the CMV promoter.

**Immunofluorescence**

Cells grown on uncoated glass coverslips (Bellco) were washed three times in PBS+ [PBS containing calcium and magnesium (each at 1.0 mM)]. The cells were then fixed with 3.7% formaldehyde for 15 min at room temperature, followed by a brief wash in PBS+ and quenching with 50 mM NH₄Cl in PBS for 5 minutes. After two further washes in PBS+, the cells were incubating in a blocking solution containing 3% BSA in PBS+. For permeabilization, the blocking and all subsequent incubation steps included 0.1% Triton X-100. Anti-TPO was diluted 1:2000 in PBS+ and was incubated with the cells overnight at 4°C. The cells were washed five times with PBS+ and then incubated with goat anti-rabbit Cy3 conjugate, diluted 1:2000, for 60 min at room temperature. The cells were finally washed five further times in PBS+ before mounting and visualization with a Nikon ECLIPSE E400 microscope equipped with epifluorescence optics and a digital camera; images were captured as Adobe Photoshop files.

**Surface Biotinylation and SDS-PAGE**

In preparation for biotinylation, cells were grown either on standard tissue-culture treated plasticware (MDCK, CHO, PC Cl3) or on Transwell-Clear filters (for MDCK cells, Costar-Corning Corp.). The cells were washed three times with ice-cold PBS+ and then incubated for 15 min with 0.6 mg/ml sulfo-NHS biotin in PBS+ at 4°C. In some experiments, two sequential 15 minute incubations with sulfo-NHS biotin were performed; however, this had no effect on the extent of surface biotinylation of hTPO nor the outcome of our experiments. After biotinylation, the cells were washed twice in ice-cold PBS+ and then quenched with 50 mM NH₄Cl in PBS for 5 minutes. For a blotting approach, the biotinylated cells were lysed at 4°C in precipitation buffer (1% Triton X-100, 0.1% SDS, 0.2% sodium deoxycholate, 100 mM NaCl, 10 mM Tris pH 7.4) plus a cocktail of protease inhibitors: aprotinin (2 ug/ml), leupeptin (100 uM), pepstatin (10 uM), EDTA (10 mM), and diisopropylfluorophosphate (1 mM). The biotinylated samples were then precipitated with avidin-agarose. Unless otherwise indicated, all of the avidin-agarose precipitate was loaded into a single gel lane, while only a portion of the supernatant was analyzed in parallel (see Figures).
samples were resolved by reducing SDS-5%-PAGE, electrotransferred to nitrocellulose, and then blotted with anti-TPO at a dilution of 1:5000.

For an immunoprecipitation approach, the cells were metabolically labeled with 300uCi [\(^{35}\)S]-amino acids, and then chased in complete growth medium. At a given chase time, biotinylated cells were lysed as above and immunoprecipitated with anti-TPO. After washing, immunoprecipitates were incubated with 100 ul boiling 1% SDS to denature the antibody. The samples were then diluted 10-fold in precipitation buffer lacking SDS and re-precipitated with avidin-agarose before analysis by reducing SDS-5%-PAGE. Gels were impregnated with 1M Na salicylate, dried, and exposed to X-ray film at -70°C.
RESULTS

Stable Expression of hTPO in clonal MDCK cells — The cloned TPO cDNA predicts a type 1 membrane protein topology with an N-terminal signal peptide [involved in protein translocation across the ER membrane], followed by a large extracellular catalytic domain, a single transmembrane span, and a short cytosolic tail at the C-terminus (30). The original reports of recombinant hTPO expression indicated the presence of a subpopulation of antibiotic-resistant CHO cells in which stably expressed, full-length wild-type hTPO933 was clearly detected on the plasma membrane (31; 32) and a truncated hTPO848 (mutated to place a stop codon that eliminates the transmembrane span and cytosolic tail, as in Fig. 1) was efficiently secreted into the medium (33-35) — both of these recombinant hTPO constructs were proved to function as enzymatically active peroxidases. Notably, Rapoport and colleagues went on to hypothesize that expression of TPO at the cell surface might create a selective growth disadvantage for cells in culture (36). In such a case, TPO-expressing cells might require mechanisms to suppress surface localization or would be outgrown during cell passage. We recently made similar observations concerning the instability in culture of surface expression of recombinant wild-type (enzymatically active) viral hemagglutinin-neuraminidase (37).

We transfected MDCK (strain II) cells with the wild-type hTPO933 cDNA in the pCDNA3 vector; selected for G418 resistance, and expanded clones for screening by Western blot. After confirming hTPO expression, a pulse-chase protocol followed by immunoprecipitation with a rabbit polyclonal antibody showed that hTPO933 in MDCK cells was relatively stable (see upper panel of Fig. 2). To initially estimate the fraction of hTPO933 molecules transported through the secretory pathway to the plasma membrane, two biochemical assays were employed. First, we used digestion with endoglycosidase H of hTPO933 immunoprecipitated from the whole cell lysate at different chase times after metabolic labeling. Endoglycosidase H digests high-mannose oligosaccharides; failure of exportable glycoproteins to be delivered to the Golgi (or failure of side chain conversion to complex glycans within the Golgi) leads to persistent sensitivity to endoglycosidase H digestion. Indeed, as shown in the lower panel of Fig. 2, the fraction of total hTPO933 acquiring resistance to digestion with endoglycosidase H in MDCK cells was undetectable. The same behavior was detected in all MDCK clones (not shown).

We then examined biotinylation of hTPO933 at the cell surface. The method involves employing a membrane-impermeant reagent that can covalently tag (with a biotin moiety) the ε-amino groups of lysine residues that are extracytoplasmically disposed and freely accessible to solvent. Fig. 3A shows the results from MDCK cells grown to confluence on porous filters, surface-tagged with
sulfo-NHS-biotin (either from the apical or basolateral side), washed and quenched, and finally lysed and precipitated with avidin-agarose to selectively sediment the biotinylated proteins. Both the precipitates and a fraction of the supernatants containing protein that had not been biotinylated were analyzed by SDS-PAGE and immunoblotting with anti-hTPO. A quantitative analysis suggested that surface hTPO in MDCK cells exhibited an apical:basolateral ratio of ~ 5 : 1 (e.g., see left panel of Fig. 3A). More significantly, however, the biotinylated surface molecules represented only ~5% of total hTPO detected (Fig. 3A). A very similar outcome was obtained when pulse labeled hTPO933 at the 6h chase time was analyzed by sequential immunoprecipitation and avidin-agarose precipitation (Fig. 3C). When sufficient amounts of surface biotinylated hTPO933 were collected for digestion with endoglycosidase H (Fig. 3A, right), even this subfraction appeared sensitive to digestion, indicating that in MDCK cells, surface hTPO933 molecules escape typical modification of their N-linked glycans upon passage through the Golgi complex, as has been previously suggested (38-40). No TPO biotinylation was detected in MDCK cells expressing truncated hTPO848 (of which no portion is localized to the cell surface, see below); this control establishes that the biotinylated hTPO933 indeed represents a subpopulation localized at the surface and not in the ER. Increased availability of heme, known to be required for recombinant TPO enzyme activity (28; 41-43) did not change either the small fraction of hTPO933 at the cell surface or the endoglycosidase H sensitivity of the biotinylated surface molecules (Fig. 3B).

We then examined the immunofluorescence localization of hTPO933 in nonpermeabilized clonal MDCK cells. Interestingly, we found that among the population of cells derived from a clone, surface expression of hTPO933 was limited to only a few (< 10%) of the cells. When observed, surface hTPO933 always showed an apical pattern of immunofluorescence, and this was especially clear in cells grown on porous filters (Fig. 4). However, in the vast majority of cells, significant hTPO933 expression became apparent only after permeabilization (Fig. 5, upper panels). Further, the permeabilized MDCK cells showed strong perinuclear and reticular fluorescence pattern throughout the cytoplasm, suggesting an ER localization.

It has been clearly shown that CHO cells extensively secrete a truncated hTPO848 (see Fig. 1) expressed from a dicistronic mRNA that includes dihydrofolate reductase for amplified recombinant gene expression (36). In our case, we introduced hTPO848 into MDCK cells using the pCDNA3 vector. However, after isolating G418-resistant colonies and then screening the medium bathing confluent cells (collected for 24 - 36h), no positive clones could be detected. We therefore went on to repeat the examination but now included analysis of the cell lysates. Fig. 6A shows 10 such clones with varying degrees of cellular hTPO848 expression. In no case was any hTPO detected in the medium by immunoblotting after a one day collection. This was true also in one of our stronger
expressors, clone HB3, even after treatment with 10 mM sodium butyrate to boost expression further (Fig. 6B). Similarly, the truncated protein could not be detected by immunoprecipitation of the medium after pulse-chase, although intracellular degradation comparable to that seen with the full-length hTPO constructed was noted (Fig. 6C). When examined by immunofluorescence, no hTPO_{848} immunofluorescence could be observed under nonpermeabilized conditions, whereas in permeabilized cells, an ER localization pattern was again detected (Fig. 5, lower panels).

**Transient expression of hTPO in CHO cells** — The foregoing experiments seemed quite consistent with recent reports indicating that only 2% of recombinant hTPO can be detected at the surface of a stably transfected clone of CHO cells (26-29). However, these findings seem at odds both with the original studies of recombinant hTPO expression in other stably transfected CHO cells (31; 33) as well as a recent suggestion of hTPO_{933} surface expression upon transient transfection of CHO cells (42). We therefore decided to reinvestigate expression of hTPO in transiently transfected CHO cells.

We began by examining the fate of the truncated hTPO_{848} construct. Importantly, nontransfected CHO cells exhibit essentially no background fluorescence when probed with anti-hTPO (see below). Using lipofectamine, we found the transfection efficiency using a green fluorescent protein cDNA in these cells to be ~10%, and immunofluorescence detection of hTPO_{848} (observed only after permeabilization) was also positive in only ~10% of the cells (not shown). We then examined a 30 h collection of conditioned medium: from clonal MDCK cells there was no secreted hTPO_{848} while transiently transfected CHO cells secreted the majority of the protein (Fig. 7A). We confirmed this result upon pulse-chase, where nearly equal concentrations of hTPO_{848} were found in the CHO cells and medium at 8 h of chase, still more was recovered in the medium at 24 h of chase (Fig. 7B), and the secreted hTPO_{848} appeared resistant to digestion with endoglycosidase H (Fig. 7B, bottom panel). These kinetics of hTPO_{848} secretion agree with those reported previously (33). As a control, a negligible fraction of full-length (membrane-bound) hTPO_{933} was found in the chase medium bathing CHO cells at the 24 h chase time (Fig. 7B, right panel). We therefore proceeded to examine hTPO_{933} expression by immunofluorescence.

In this case, ~10% of the cells were brightly immunofluorescent when probed with anti-hTPO under nonpermeabilized conditions (Fig. 8, left panels). Incidentally noted was the tendency of hTPO_{933}-expressing CHO cells to assume a slightly more elongated shape than surrounding nontransfected cells; the significance of this observation is unclear. Individual nonpermeabilized hTPO_{933}-positive CHO cells also exhibited immunofluorescence that tended to be brighter near the cell edges (where the cells are more flat) than over the cell mid-portion, which often had a darker
central region (Fig. 8, left panels). Upon permeabilization, the fraction of hTPO933-positive cells did not change and the immunofluorescence intensity was not increased over that of nonpermeabilized cells (Fig. 8, right panels). This result was repeated in several independent experiments. CHO cells are notably poor for high resolution analysis of intracellular organelle staining patterns by conventional immunofluorescence; thus, one cannot readily quantify the extent to which ER and/or Golgi staining can be superimposed upon the cell surface staining that is already apparent in the nonpermeabilized cells. Further, the biotinylation assay did not seem particularly advantageous in this case where the vast majority of cells are not expressing the hTPO933 protein. However, the immunofluorescence data make clear that surface expression of hTPO933 in transiently transfected CHO cells is much higher than in clonal MDCK cells (see above) or in selected clones of CHO cells (26-29). Since both our study and those reported previously (26-29) used the identical source of hTPO933 cDNA for expression in the identical (CHO) cell type, the clear implication of these results is that upon selection, expansion, and maintenance of clones, some (and perhaps even most) clones are eventually propagated whose surface hTPO expression is no longer representative of the initial behavior of hTPO933 in CHO cells.

Stable expression of hTPO in the PC Cl3 (rat thyrocyte) cell line — Because of the remarkably inefficient delivery of hTPO to the surface of stable clones of CHO and MDCK cells, we decided to examine the surface expression of hTPO933 in a cell line derived from a thyrocyte lineage. PC Cl3 cells are a rat thyrocyte line similar in origin to FRTL5 cells that contain endogenous rTPO (20; 44) and have served as a suitable expression system for hTPO (45). When probed by Western blotting, there was no detectable hTPO cross-reactivity in untransfected PC Cl3 cells (Fig. 9A, lane 9). Eight independent G418-resistant clones of transfected PC Cl3 cells were selected and screened by immunoblotting for expression of the hTPO933 cDNA (Fig. 9A, lanes 1-8). Three clones, C5 (lane 1), C4 (lane 6) and B6 (lane 7) were found to be positive and were studied further.

When hTPO933 surface expression was examined by immunofluorescence in nonpermeabilized clonal cells, it was again surprising that not all cells in the population showed the same pattern. Specifically, about half the nonpermeabilized cells were bright while the other half exhibited no surface fluorescence (Fig. 10). Importantly, this pattern was observed in each of the three expressing clones. The surface hTPO appeared to be distributed in a dense punctate pattern (Figs 11 and 12). Thus, while the frequency of surface-positive hTPO933 expression in stable clones of PC Cl3 thyrocytes (~50%) was not as high as in transiently transfected cells (where it appeared to approach 100%, see Fig. 8), it was clearly much higher than in stably transfected MDCK cells (<10%, see Figs 4 and 5). Moreover, the absence of surface immunofluorescence from the other portion of PC Cl3 cells was not due to a lack of hTPO933 protein expression, as every cell in the
population in these clones was positive for hTPO after permeabilization (Fig. 11, right panels) while immunofluorescence could not be detected in untransfected, permeabilized PC Cl3 cells (not shown). Permeabilization primarily increased the fraction of positive cells rather than the immunofluorescence intensity of the cells. Nevertheless, in the permeabilized cells an ER localization pattern was also suggested in addition to the surface fluorescence (Fig. 11). Together, these data clearly indicate a markedly increased distribution of recombinant hTPO

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DISCUSSION

TPO has been a protein of special interest to thyroid biologists for the past twenty years. At this point, the evidence is overwhelming that this type 1 membrane protein is delivered to the apical plasma membrane of thyrocytes via the secretory pathway, and abnormal subcellular distribution of mutant hTPO may be associated with congenital hypothyroid goiter (42; 46). However, the fraction of cellular TPO that normally resides on the plasma membrane — a fraction which is clearly regulated by cell type specific factors, including TSH stimulation (18-24) — has been an area of confusion. Several technical limitations have compromised the analysis of hTPO trafficking. First, TPO may be modified by Golgi processing enzymes in some systems [(26) and Fig. 7B of this report], not in others (38-40), and only partially modified in still other systems (Fig. 9B of this report). Indeed, after TPO folding, it is not clear that the glycans themselves are needed for enzymatic activity or immunoreactivity, although it is difficult to deglycosylate the native protein which suggests that the glycans are relatively inaccessible in the tertiary structure (38; 47-49). Second, most antisera used to examine the intracellular distribution of thyroidal TPO (e.g., by immunofluorescence) are derived from patients who may have antibodies that simultaneously recognize other thyroid antigens.

Some of these problems may be circumvented by examining the expression of recombinant hTPO in heterologous cell types. However, there are additional technical limitations that must be considered. Notably, cell surface biotinylation requires that the protein tertiary structure allows lysines in the extracellular domain to be freely accessible to the reactive N-hydroxysuccinimidyl group (50). It cannot automatically be assumed that all membrane proteins under standard conditions will exhibit equally such reactivity (51), especially proteins like hTPO that have a complex tertiary structure (52). Indeed, in pilot studies in our laboratory (not shown), we found that when hTPO933-expressing cells were lysed before biotinylation, the efficiency of hTPO 933 recovery by precipitation with avidin-agarose varied between experiments in a manner that appeared to depend upon detergent conditions, suggesting that the native conformation of the hTPO933 protein may limit its biotinylation efficiency. As a consequence, we have been careful to independently examine each of our cell culture systems by immunofluorescence with anti-hTPO under nonpermeabilized and permeabilized conditions. From a combination of these analyses, we now report that there are major cell-type dependent differences in the handling of the same hTPO construct in different stably transfected mammalian cell types. Further, these differences appear to follow as a direct consequence of heterogenous cell surface expression that occurs even within a clonal population in which all cells are positively expressing hTPO as measured under permeabilized conditions (Figs. 4, 5, 11, 12).
First, in MDCK cells, we conclude that the hTPO933 that can be biotinylated at the cell surface shows a predominantly apically polarized distribution; however, most hTPO933 cannot be biotinylated and does not acquire endoglycosidase H resistance (Fig. 3). Instead, cell surface hTPO933 in MDCK cell clones comes from a small fraction of cells that exhibit a relatively high level of surface expression (Fig. 4) while the remaining hTPO933-positive cells express little or none at the cell surface (Fig. 5).

Second, in CHO cells, we conclude that the observations recently reported from one stable clone (26-29) are not representative of the very high hTPO trafficking to the cell surface that is initially obtained from these cells [(42) and Fig. 8 of this report]. The data indicate that the problem is not a defect of protein expression or an intrinsic defect in the secretory pathway to accommodate hTPO. Rather, the data suggest that upon growth and expansion of hTPO933-positive clones, an evolution in the cell population takes place such that many [but perhaps not all (31; 32)] clones can be isolated in which hTPO fails to be efficiently delivered to the cell surface (26-29). This would be consistent with a tendency towards morphological changes in the cells (Fig. 8) and a growth disadvantage for cells in the population that exhibit high cell surface hTPO delivery. Indeed, nearly 10 years ago, McLachlan and Rapoport postulated that there might be a growth disadvantage for certain cell cultures that externalize recombinant hTPO (36). We believe that the present observations support such an interpretation. Certainly, these data raise questions about: a) whether an individual clone can be considered representative of a population of transfected cells, and more importantly, b) whether results from such clones can be used to reflect physiological mechanisms of hTPO regulation, such as targeting for intracellular degradation (26-29).

Finally, from our observations in clonal PC C13 cells, we conclude that the (rat) thyroid lineage may be more resistant to loss of cell surface hTPO933 expression over time than are MDCK or CHO cells. Thus, thyrocytes may be to some extent growth-adapted to surface TPO expression. Nevertheless, even with continuous antibiotic selection pressure, and persistent hTPO expression in the entire population of permeabilized cells (Fig. 11), only about half the cells in our clones exhibited detectable surface at a moment in time in the steady state (Fig. 10). In this context, it is interesting to note a recent report that by immunofluorescence in thyrocytes after 18 d in primary culture, ≤5% of TPO is detected at the plasma membrane (24). Although such prolonged primary thyrocyte culture has not been extensively characterized, almost certainly these cells have an extremely low mitotic index. More study is needed to determine: a) whether the same fraction of surface hTPO933 can be maintained in a clonal population (such as the PC C13 thyrocytes we have described) even after extensive passaging (e.g., for a year or more); b) whether hTPO surface...
expression in thyrocytes might appear at different stages of the cell cycle, in which case, conceivably, all cells in the PC Cl3 line (eg., Figs. 10, 11) might exhibit surface expression at different times; c) whether endogenous rTPO has a similar distribution in PC Cl3 cells (no good antibody to rTPO currently exists), and d) whether surface expression of bioactive hTPO is selectively inhibitory to any particular stage of the cell cycle or whether it induces cell death.

Taken together, these results highlight previously unappreciated complexity in the intracellular transport of hTPO in selected cell culture systems, and suggest that cell-type specific factors may stabilize plasma membrane expression of hTPO in thyrocytes.
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Figure Legends

Figure 1. Schematic of hTPO cDNAs expressed in this paper. Sig = cleaved signal peptide; TM = transmembrane span; C = cytosolic tail domain. The arrow above identifies the location of residue 848 at the beginning of the transmembrane span; truncation at this position in the construct below has been reported to produce a secreted, bioactive hTPO (ref. 33).

Figure 2. Stability and sensitivity to endoglycosidase H (Endo H) of recombinant hTPO933 in stably transfected MDCK cells. Confluent cells grown in 6-well plates were pulse labeled with [35S]-amino acids for 30 min and then individual wells of cells were lysed at each of the chase times indicated, immunoprecipitated with a rabbit polyclonal antiserum against hTPO, and analyzed by SDS-PAGE and fluorography. To be representative, two different clones (upper panel: clone 2a; lower panel: clone 3c) are shown in the Figure. The same patterns shown here were observed in all MDCK clones tested.

Figure 3. Cell surface biotinylation of hTPO933 expressed in MDCK cells cultured on porous filters. MDCK cells (clone 3c) were grown for 3 days on Transwell-Clear filters. At this time transepithelial resistances of ≥ 100 ohm-cm² were confirmed. The cells were then biotinylated either apically or basolaterally as described in Experimental Procedures. A: Apically (A) and basolaterally (B) biotinylated proteins bound to avidin-agarose were detected by immunoblotting with anti-hTPO, in comparison to one-tenth of the avidin-unbound supernatant. In control experiments, re-precipitation of the supernatant with additional avidin-agarose recovered no additional hTPO, while precipitation of non-biotinylated cell lysates with avidin-agarose also recovered no hTPO (not shown). In the right panel of A: biotinylated hTPO933 from additional MDCK cells was mock-digested (-) or digested (+) with endoglycosidase H (Endo H). B: The same experimental protocol was repeated but this time including 30 μM hemin in the medium throughout the experiment. In this case, only one-twentieth of the avidin-unbound supernatant was analyzed on the gel. Note that increased heme availability did not appreciably increase the avidin-bound fraction of hTPO933, which was again sensitive to digestion with endoglycosidase H. C: The cells were pulse-labeled as in Fig. 2 and chased for 6h before surface biotinylation from either apical (A) or basolateral (B) sides. Total labeled hTPO933 at the 6h chase time from a parallel filter is shown for comparison.

Figure 4. Surface hTPO expression in MDCK cells (clone 3c) grown on Transwell-Clear filters. The cells were processed for immunofluorescence with a polyclonal anti-hTPO under nonpermeabilized conditions (upper panel) and counterstained with DAPI (middle panel) to identify
nuclei. The lower panel is a merged view. Note that most nonpermeabilized cells exhibit no surface immunofluorescence.

Figure 5. Immunofluorescence distribution of full-length hTPO$_{933}$ and truncated hTPO$_{848}$ in MDCK cells. MDCK cells expressing hTPO$_{933}$ (clone 3c, upper panels) or hTPO$_{848}$ (clone HB3, lower panels), grown on glass coverslips, were processed for immunofluorescence with anti-hTPO under nonpermeabilized or permeabilized conditions as indicated.

Figure 6. Lack of secretion of truncated hTPO$_{848}$ from different MDCK cell clones. A: For each of 10 different confluent G418-resistant clones of MDCK cells transfected with the hTPO$_{848}$ cDNA, growth medium was collected for one day. The media were TCA precipitated, cells lysed, and both were analyzed by immunoblotting with anti-hTPO. B: Clone HB3, one of our highest expressors, was grown overnight in the presence or absence of butyrate to further increase hTPO$_{848}$ expression, but this did not lead to detectable secretion. C: After pulse labeling with $[^{35}\text{S}]-$amino acids, clone HB3 was chased for 0.5 h or 6 h and the cell lysates and medium analyzed by immunoprecipitation with anti-hTPO, SDS-PAGE, and fluorography. Some decrease in intracellular hTPO$_{848}$ is apparent over this time, but no hTPO$_{848}$ is detected in the medium.

Figure 7. Truncated hTPO$_{848}$ is efficiently secreted from transiently transfected CHO cells. A: Beginning at 48h post-transfection, conditioned medium was collected for 30 h from transiently transfected CHO cells or, as a control, from stably transfected MDCK cells (clone HB3). The cells were lysed and media TCA precipitated, before analysis by SDS-PAGE and immunoblotting. In the first lane ("Con"), MDCK cells expressing full-length hTPO$_{933}$ were analyzed as positive control. B: After pulse labeling with $^{[35}\text{S}]-$amino acids, CHO cells transiently transfected to express truncated hTPO$_{848}$ (left part of Figure) or full-length hTPO$_{933}$ (right part of Figure) were chased for 8 h or 24 h and the cell lysates (C) and media (M) were analyzed by immunoprecipitation with anti-hTPO followed by SDS-PAGE and fluorography. C: Endoglycosidase H digest of cellular and secreted hTPO$_{848}$ at the 8 h chase time.

Figure 8. Survey of the population of transiently transfected CHO cells indicates that full-length hTPO$_{933}$ is strongly expressed at the cell surface. The cells at 48h post-transfection were processed for immunofluorescence with anti-hTPO under nonpermeabilized or permeabilized conditions. As indicated, the upper left panel demonstrates the degree of background fluorescence from a population of nontransfected CHO cells. Note that, unlike in MDCK cells (see Fig. 5), the frequency of positively expressing cells is equal under nonpermeabilized and permeabilized conditions.
Figure 9. Western blot screening, cell surface biotinylation, and endoglycosidase digestion of PC Cl3 cells stably transfected with the cDNA encoding hTPO933. **A:** In the Western blot, lanes 1, 6, and 7 were positive for hTPO933 expression and were designated with the clone names shown above. Lane 9 shows untransfected PC Cl3 cells. Lane 10 shows MDCK cells expressing hTPO933 as a positive control. **B:** Clone B6 cells were biotinylated, precipitated with avidin-agarose, and analyzed as in Fig. 3A, except that the biotinylated fraction was divided in three equal portions and digested with PNGase F, endoglycosidase H, or mock-digested. Two different concentrations of the avidin-unbound fraction (one-fiftieth and one-twentieth) were loaded side-by-side. From these data, we estimate that ≤15% of hTPO molecules can be detected at the surface by this method, and this is probably an underestimate (see text). Moreover, the population of surface-biotinylated TPO is largely sensitive to digestion with endoglycosidase H, in agreement with published reports (39).

Figure 10. Heterogeneity of cell surface expression of hTPO933 in stably transfected PC Cl3 cells. The upper panel shows an island of transfected PC Cl3 cells (clone B6) by phase-contrast microscopy. In the middle panel, the same cells were processed for immunofluorescence with anti-hTPO under nonpermeabilized conditions. The lower panel is a merged view. In multiple such samplings, approximately half the cells are strongly positive for cell surface immunofluorescence under nonpermeabilized conditions.

Figure 11. Survey of hTPO933 expression PC Cl3 cells. The cells were processed for immunofluorescence with anti-hTPO under nonpermeabilized or permeabilized conditions. Upper panels: clone C4. Lower panels: clone C5. Note that both of the left-sided fields each show one continuous island of cells. The discontinuities of immunofluorescence in these islands represent cells with little or no surface expression of hTPO933 — in multiple such samplings, approximately half the cells in these clones are strongly positive for cell surface immunofluorescence under nonpermeabilized conditions. However in the right-sided fields, all PC Cl3 cells are positive for hTPO933 expression — discontinuities of fluorescence reflect only the extent to which the cells are subconfluent.
| Sig | TPO catalytic domain | TM | C | wt |
|-----|----------------------|----|---|----|
|     |                      | 828|    |    |

| Sig | TPO catalytic domain | truncated |
|-----|----------------------|-----------|
|     |                      | 828       |

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