Human Thyroperoxidase in Its Alternatively Spliced Form (TPO2) Is Enzymatically Inactive and Exhibits Changes in Intracellular Processing and Trafficking*

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Thyroid peroxidase (TPO1) is a membrane-bound heme-containing glycoprotein that catalyzes the synthesis of thyroid hormones. We generated stable cell lines expressing TPO1 and the alternatively spliced isoform TPO2. Pulse-chase studies showed that TPO2 half-life was dramatically decreased as compared with TPO1. The sensitivity of TPO2 to endo-β-N-acetylglucosaminidase H indicated that the protein is processed through the endoplasmic reticulum and bears high mannose-type structures. Cell surface biotinylation experiments showed that the two isoforms also differ in their intracellular trafficking. TPO2 was totally retained in the cell, whereas 15% of TPO1 reached the cell surface. The inability of TPO2 to come out of the intracellular compartments was related to structural changes in the molecule. Evidence of these changes was obtained through the lack of recognition of TPO2 by half of the 13 TPO monoclonal antibodies tested in immunoprecipitation experiments. Our data suggest that because of an improper folding, TPO2 is trapped in the endoplasmic reticulum and rapidly degraded. The failure of incorporation of [14C]aminolevulinic acid in the cultured cells showed that TPO2 did not bind to heme, whereas TPO1 did. This result was confirmed through a guaiacol assay showing that TPO2 is enzymatically inactive.

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* The abbreviations used are: TPO, thyroperoxidase; CHO, Chinese hamster ovary; mAb, monoclonal antibody; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; NHS-SS-biotin, sulfosuccinimidyl-3′-(biotinamido)ethyl-1,3-dithiopropionate; ER, endoplasmic reticulum.

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The sensitivity of TPO2 to endo-β-N-acetylglucosaminidase H indicated that the protein is processed through the endoplasmic reticulum and bears high mannose-type structures. Cell surface biotinylation experiments showed that the two isoforms also differ in their intracellular trafficking. TPO2 was totally retained in the cell, whereas 15% of TPO1 reached the cell surface. The inability of TPO2 to come out of the intracellular compartments was related to structural changes in the molecule. Evidence of these changes was obtained through the lack of recognition of TPO2 by half of the 13 TPO monoclonal antibodies tested in immunoprecipitation experiments. Our data suggest that because of an improper folding, TPO2 is trapped in the endoplasmic reticulum and rapidly degraded. The failure of incorporation of [14C]aminolevulinic acid in the cultured cells showed that TPO2 did not bind to heme, whereas TPO1 did. This result was confirmed through a guaiacol assay showing that TPO2 is enzymatically inactive.

Thyroid peroxidase (TPO) is the major enzyme in the biosynthesis of thyroid hormones, because it catalyzes the iodination and coupling of iodotyrosine residues on thyroglobulin to produce thyroxine (T4) and 3,3′,5′-triiodothyronine (1–3). TPO is a major autoantigen in the pathogenesis of autoimmune thyroid diseases (4–6). Also, defective TPO causes congenital hypothyroidism because of an iodide organization defect linked with mutations of the TPO gene (7–9).

TPO is a membrane-bound glycoprotein of 933 amino acids containing a heme prosthetic group. Kimura et al. suggested that His-407 or His-414 is the proximal heme binding site (10). On the other hand, His-586 is conserved in human and porcine TPO and also in human myeloperoxidase (11), which suggests that His-586 may be a critical residue for the enzymatic activity of the protein by being the putative distal heme binding site. Four potential asparagine-linked glycosylation sites are also present in the extracellular region, and there is some evidence for at least one disulfide bond in this part of the molecule (12).

Distribution and delivery of TPO are apically polarized, but only 30% TPO is detected at the thyrocyte surface (13). Mistargeting of another small fraction of TPO to the basolateral surface seems to occur (13, 14). This may account for the hypothetical presentation of TPO to the circulating immune system, resulting in its antigenicity and its part in autoimmune thyroid diseases. In the presence of H2O2, TPO catalyzes the iodination of thyroglobulin, which is processed mainly at the apical membrane of the thyrocyte (15–17).

However, a quantitatively minor iodination of thyroglobulin appears to occur intracellularly (18). TPO gene codes for the full-length TPO (TPO1) and generates alternatively spliced forms (for review see Ref. 5). The main alternatively spliced TPO mRNA species described so far has a 171-base pair deletion ( exon 10) and codes for a protein of 876 amino acids (TPO2) (19, 20). TPO2 is 57 amino acids shorter than the major protein and lacks one of the potential glycosylation sites and His-586. TPO2 mRNA has been detected in normal and Graves thyroid (19, 20) but also in a case of congenital hypothyroidism caused by a premature termination signal in the TPO gene, suggesting that TPO2 is enzymatically inactive (21). Note that 10 years after the discovery of TPO2-specific mRNA, nothing is known about the protein. Hence no further information is available on its enzymatic and immunological activities.

Using CHO cell lines expressing TPO2 and TPO1, we investigated the structural and functional aspects of TPO2. With reference to the major protein TPO1, we determined how changes of the primary sequence affected the conformational structure of the protein, as shown through its epitopic recognition by TPO mAbs. We determined the effects of those structural modifications on the intracellular trafficking, the localization, and the enzymatic activity of the protein.

** MATERIALS AND METHODS **

** Construction of pcDNA3-TPO2—** Full-length 3060-kilobase human TPO1 cDNA kindly provided by B. Rapoport, University of California, San Francisco, CA, was cloned into HindIII and XbaI sites of the eukaryotic transfer vector pcDNA3 (Invitrogen, Leek, The Netherlands). A 578-nucleotide cDNA fragment corresponding to a region of TPO2 (1411–1989 base pairs) around the splicing was amplified by polymerase chain reaction from the λgt11 human thyroid cDNA library (22). Oligonucleotides FP, 5′-TATGATTCTATGACTCCACCGCC, and RP, 5′-GTCTCTAGACTCAAGTACCTT, were used as primers (23). This 578-base pair-amplified fragment containing at both extremities the NcoI restriction site was cleaved by this enzyme. TPO1 cDNA was then released from TPO1-pcDNA3 construction and subcloned in pBluescript (Stratagene, La Jolla, CA). TPO1-pBluescript was then cleaned of the 748-base pair fragment containing the sequence that corresponds to exon 10 by double cleavage with NcoI and dephosphorylated by alkaline phosphatase.
phosphatase. TPO1-pBluescript and the TPO2-NcoI fragment were then ligated. TPO2-pBluescript construct was cleaned of the TPO2 cDNA, which was cloned into HindIII and XbaI sites of the eukaryotic transfer vector pcDNA3. The nucleotide sequence of the polymerase chain reaction-derived NcoI fragment was determined by dideoxynucleotide chain termination to verify that the engineered linker fragment was correct. Escherichia coli strain XL1 Blues (Stratagene) was transformed with the TPO2-pcDNA3 construct, and pure plasmid DNA preparations were obtained with the Wizard midiprep kit (Promega, Madison, WI). Enzyme reactions and DNA manipulations were done as in Sanbrook et al. (24).

**RESULTS**

**Coimmunoprecipitation and Transfection**—CHO cells (ECACC n°8505002) were maintained in Ham's F-12 medium supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Cells were transfected by the lipofectAMINE method (Life Technologies, Inc.) with either pcDNA3-TPO1 or pcDNA3-TPO2 or pcDNA3 alone as control. Cells were cultured in a humidified incubator (5% CO2) at 37 °C. Stable transfectants were selected in the presence of genetin (400 μg/ml) and were subcloned by limiting dilutions. Positive TPO-expressing cell lines were identified by Western blotting or by immunoprecipitation after [35S]Met + [35S]Cys labeling (Expression Systems) and, in some cases, by recombinant protein labeling mix, DuPont NEN Life Science Products. A significant TPO1 and TPO2 expression was observed by culturing CHO TPO cell lines with 10 μM sodium butyrate.

**Heme and Immunoprecipitation**—Cells were incubated in cysteine- and methionine-free Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 mM sodium butyrate, and 100 μCi/ml [35S]Met + [35S]Cys. Incubation was done for 5 or 48 h. In pulse-chase experiments, cells were incubated 1 h in Cys- and Met-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed FBS and 10 mM sodium butyrate. Cells were then pulse-chased for 30 min in the presence of 100 μCi/ml [35S]Met + [35S]Cys. After the pulse, the labeling medium was removed, and the cell surface was washed three times with PBS and then replaced by Dulbecco's modified Eagle's medium supplemented with 10% FBS, 5 mM Met, and 5 mM Cys. Chases were performed for 1, 5, 22, and 48 h and 72 h.

**Cell Lysis and Immunoprecipitation**—After being metabolically labeled, cells were harvested on ice by scraping in 1 ml of PBS and centrifuging at 700 × g for 7 min. Cell pellets were resuspended in 60 μl of TPO extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.3% sodium deoxycholate, protease inhibitors (Complete*), Boehringer Mannheim), vortexed every 2 min during 20 min, and centrifuged at 10,000 × g for 5 min. Radiolabeled supernatants were incubated for 2 h at room temperature with mAbs recognizing either a sequential region (mAb 47) or conformational epitopes (mAb 15 and mAb 53) on the surface of the TPO molecule (25). mAbs 47 and 53 were used for TPO2, and mAbs 47 and 15 were used for TPO1. The use of two TPO mAbs in immunoprecipitation experiments allowed us to recover more than 90% of both TPO1 and TPO2 expressed by CHO cells (data not shown). mAbs were chosen for their strong recognition of each isoform (see "Results") through a panel of TPO mAbs. These mAbs were prepared by a hybridoma supernatant (Zymed Laboratories Inc., San Francisco, CA) by incubation overnight at 4 °C. Immune complexes were then retrieved by a brief centrifugation (10,000 × g, 10 s) and were washed 6 times with 1 ml of TPO extraction buffer and once with 1 ml of PBS. Immunoprecipitated TPO was recovered from mAb-protein A-Sepharose 4B complexes by boiling for 5 min in 80 μl of 10% SDS, diluted in the incubation buffer with either endo-β-N-acetylglucosaminidase H or peptide-N-glycanase F, centrifuged (10,000 × g, 10 s), and boiled for 5 min. Treatment with 0.2 unit of endo-β-N-acetylglucosaminidase H (Boehringer Mannheim) was done in 0.15 M sodium acetate buffer, pH 5.0, for 16 h at 37 °C. Deglycosylation by 1 unit of peptide-N-glycanase F (Boehringer Mannheim) was done in 0.15 M phosphate buffer, pH 8.5, for 16 h at 37 °C. Samples were then analyzed by SDS-PAGE.

**Surface Biotinylation**—CHO TPO1 and CHO TPO2 confont mono-layers were metabolically labeled for 16 h with 100 μCi/ml [35S]Met + [35S]Cys in the presence of 10 mM sodium butyrate and culture surface-biotinylated as in Ref. 26. Cells were washed twice with PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2 and exposed to a 0.5 mg/ml Immunopure NHS-SS-biotin (Pierce) 20 min at 4 °C. Cross-linker was removed, and the same procedure was repeated once. The biotin reagent was quenched by incubation with 50 mM NH4Cl in PBS for 10 min at 4 °C. Cells were washed with PBS and harvested. To recover the immunoprecipitated antigens, we supplemented the complexes with 10 μl of 1 M NaCl, boiled them with 600 μg TPO extraction buffer, and centrifuged (10,000 × g, 3 min). Supernatant containing total TPO was incubated 2 h with avidin-agarose (Pierce). Biotinylated surface TPO and intracellular TPO were separated by centrifugation (10,000 × g, 3 min). The beads were washed four times with TPO extraction buffer and once with PBS, resuspended in electrophoresis buffer, and boiled for 5 min. The supernatants were analyzed by SDS-PAGE.

**Heme Binding to TPO**—Cells were incubated in Ham's F-12 medium supplemented with 10 mM sodium butyrate, 1% FBS, and 5 μg/ml [14C]aminolevulinic acid (NEN Life Science Products) for 16 or 48 h. Cells were then harvested, and TPO was immunoprecipitated as described previously before being analyzed by SDS-PAGE.

**Microsomal Fractionation**—Cells were harvested in the presence of 10 mM sodium butyrate for 48 h and washed once with PBS. Cells were harvested by scraping in PBS and centrifuged at 200 × g for 7 min. Pellets were resuspended in 15 mM Tris-HCl, pH 7.4. Cells were ruptured by a Teflon glass homogenizer, and the suspension was centrifuged for 1 h at 110,000 × g.

**TPO Enzymatic Activity**—Microsomal fraction pellets were solubilized by nuclease 450 μg/ml (Fluka Chimie, St. Quentin-Fallavier, France) and 67 mM sodium phosphate buffer, pH 7.5. The reaction was done at room temperature and initiated by adding H2O2 to reach a final concentration of 0.25 mM. Guaiacol oxidation was measured by absorbance at 470 nm and spectrophotometrically monitored every 30 s for 3 min. Variation of absorbance for each assay was calculated on the basis of a mean of three experiments.

**RESULTS**

**Expression of TPO2 in CHO Cell Lines**—To investigate the structure-function relations and the intracellular trafficking of TPO2 isoform as compared with the major variant TPO1, we generated two CHO cell lines stably expressing TPO2 and TPO1. CHO cells were transfected with either TPO2-, TPO1-pcDNA3 constructs or with pcDNA3 alone as control. After obtaining several clones expressing TPO2 and TPO1, we did the experiments in the presence of 10 mM sodium butyrate, which made TPO2 expression in the immunoprecipitation levels higher than those performed without butyrate addition (data not shown).

After metabolic labeling of CHO TPO cells with [35S]Met + [35S]Cys for 5 h, we immunoprecipitated TPO2 and TPO1 with a combination of two TPO mAbs. As expected (28), TPO1 gave a 110-kDa specific band (Fig. 1, lane A), whereas TPO2 was a 98-kDa band on SDS-PAGE analysis (Fig. 1, lane B). The difference in apparent molecular mass between TPO1 and
TPO2 isoforms reflects the lack of 57 amino acids in the extracellular domain of the TPO2 molecule. This difference may also be explained by the loss of a potential asparagine N-linked glycosylation site (Asn-569). These two bands were shown to specifically represent TPO1 and TPO2 isoforms, as they correspond to a binding with TPO mAbs and were not present in CHO cells transfected with pcDNA3 alone (Fig. 1, lane C). Similar results were obtained by Western blotting analysis of TPO1 and TPO2 with mAb 47 as first antibody (data not shown).

During the subcloning of the transfected cells, we constantly found a higher amount of TPO1 than of TPO2 in the clones. Within the selected clones, quantification of each of the TPO band intensities after either steady-state labeling with [35S]Met + [35S]Cys for 48 h or Western blotting revealed a strong difference between TPO1 and TPO2 levels recovered. We always recovered 25-fold less TPO2 than TPO1. We believe this is due to the lower stability of the glycoprotein, we did pulse-chase studies and determined the half-life of TPO1 and TPO2. Cells were pulsed for 30 min in the presence of [35S]Met + [35S]Cys and chased at various times. Immunoprecipitated TPO1 and TPO2 were anti-

We then determined TPO2 immunological reactivity against a panel of mAbs directed to TPO1 (25). After [35S]Met + [35S]Cys metabolic labeling for 5 h, TPO1 and TPO2 were tested by immunoprecipitation with each of the 13 TPO mAbs. TPO1 immunoreactivity was observed with all the mAbs tested (Fig. 3A). For TPO2, a strong recognition was observed with mAb 47 and mAb 53, which led us to use these mAbs in immunoprecipitation experiments on TPO2 (Fig. 3B). Four other mAbs also clearly recognized TPO2 (mAbs 1, 15, 24, and 30), although 7 out of 13 had slight or no reactivity to the protein (Fig. 3B). TPO2 exhibited either a weaker or no immunoreactivity to some of the mAbs suggests that the molecule changed conformation.

Given these structural changes, we questioned whether the TPO2 molecule is still recognized by TPO autoantibodies. The 57-amino acid region deleted in TPO2 is upstream from the main epitope recognized by autoantibodies, but the conformational changes previously deduced may be expected to modify the exposure of some autoepitopes. We thus determined the antigenic immunoreactivity of TPO2 against sera of patients with high titers of TPO autoantibodies. Using immunoprecipitation experiments, we showed that serum TPO autoantibodies specifically bound TPO2 (Fig. 4, lanes 2 and 3), whereas the serum of normal individuals did not (Fig. 4, lane 4). This suggests that TPO2 remained recognized by autoantibodies and exhibited all or part of the autoepitopes at the surface of the molecule.

**FIG. 1. Immunoprecipitation of [35S]Met + [35S]Cys labeled TP01 and TPO2 from stably transfected CHO cell lines.** Cells were incubated with [35S]Met + [35S]Cys for 48 h and then lysed. TPO1 and TPO2 were immunoprecipitated with mAb 47 + mAb 15 for TPO1 (lane A) and mAb 47 + mAb 53 for TPO2 (lane B). CHO cells transfected with the eucaryotic vector pcDNA3 were used as control (lane C). Samples were run on 7.5% SDS-polyacrylamide gels, and the bands were visualized by phosphoimager.

**FIG. 2. Enzymatic deglycosylation of TPO2.** CHO TPO2 cells were metabolically labeled with [35S]Met + [35S]Cys for 5 h. Labeled TPO2 was immunoprecipitated with mAb 47 + mAb 53 as described previously before being eluted from the immunocomplexes and digested with endo-β-N-acetylglucosaminidase H (lane 2) or 1 unit of peptide-N-glycanase F (lane 3). Incubation without deglycosylating enzymes served as control (lane 1). Samples were then analyzed by 7.5% SDS-PAGE, and the bands were visualized by phosphoimager. Upper and lower arrows indicate the glycosylated and deglycosylated forms of TPO2, respectively.

**FIG. 4. Analysis of TPO2 immunoreactivity against TPO autoantibodies.** TPO2 was tested against TPO autoantibodies in the sera from two patients with Hashimoto’s thyroiditis (lanes 2 and 3). Serum of a normal individual (lane 4) and TPO2 immunoprecipitated with mAb 47 + mAb 53 (lane 1) were used as controls. Radiolabeled TPO2 immunoprecipitated by autoantibodies was eluted from complexes and analyzed by 7.5% SDS-PAGE.

**FIG. 3. Analysis of TPO1 and TPO2 immunoreactivity against TPO-mAbs.** TPO1 (A) and TPO2 (B) were immunoprecipitated with a panel of mAbs directed against various antigenic domains of the TPO1 molecule (25). CHO TPO cells were incubated with [35S]Met + [35S]Cys for 5 h. For TPO1 immunoprecipitation, one-third of a 9.6-cm dish was used, whereas for TPO2, immunoprecipitation of eight dishes of 9.6-cm² was used. TPO was recovered from mAb-protein A-Sepharose 4B complexes and then analyzed by SDS-PAGE (7.5%). Protein-associated radioactivity was visualized by phosphoimaging.

**Kda**

![Kda](image)

1 2 3

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Fig. 5. Rate of degradation of TPO1 and TPO2. CHO TPO cells were pulsed for 30 min in the presence of 100 μCi/ml [35S]Met + [35S]Cys. After the pulse, the labeling medium was removed and replaced by Dulbecco's modified Eagle's medium supplemented with unlabeled amino acids. At the times indicated, radiolabeled cell lysates were immunoprecipitated with mAb 47 for TPO1 (A) and mAb 47 + mAb 53 for TPO2 (B). Immunoprecipitated TPO was analyzed by SDS-PAGE and visualized by phosphoimaging. The arrows indicate TPO1 or TPO2 bands identified in each experiment. Each labeled amino acids. At the times indicated, radiolabeled cell lysates were immunoprecipitated with mAb 47 for TPO1 (A) and mAb 47 + mAb 53 for TPO2 (B). Immunoprecipitated TPO was analyzed by SDS-PAGE and visualized by phosphoimaging. The arrows indicate TPO1 or TPO2 bands identified in each experiment. Each labeled band of TPO1 (●) and TPO2 (○) was quantitated by phosphoimaging (C).

Fig. 6. Cell distribution of TPO1 and TPO2. Cells were metabolically labeled for 16 h with [35S]Met + [35S]Cys. Cell surface biotinylation was done as described under "Materials and Methods." TPO1 and TPO2 were immunoprecipitated as described from cell lysates and then reprecipitated with avidin-agarose. The avidin-unbound (intracellular) and avidin-bound (surface tagged) fractions were analyzed by SDS-PAGE. Radiolabeled TPO1 (lanes 1, 2, 5, and 6) and TPO2 (lanes 3, 4, 7, and 8) were visualized by phosphoimaging. Each experiment was done with and without NHS-SS-biotin reagent (-).

Fig. 7. Incorporation of [14C] aminolevulinic acid into CHO TPO cells. Cells were incubated in Ham's F-12 medium supplemented with 10 mM sodium butyrate, 1% FBS, and [14C]aminolevulinic acid for 16 h (lanes 1 and 2) or 48 h (lanes 3 and 4). TPO1 and TPO2 were then immunoprecipitated as described and analyzed by SDS-PAGE. [14C]-radiolabeled TPO1 (lanes 1 and 3) and TPO2 (lanes 2 and 4) were visualized by phosphoimager.

with avidin-agarose. This procedure separated biotinylated surface TPO bound to avidin-agarose from intracellular TPO, which is hence inaccessible to the tagging agent. TPO1 was found predominantly in the intracellular compartments; a small fraction was present at the cell surface (Fig. 6, lanes 2 and 6). Quantification of the bands revealed that only 15% of TPO1 reached the cell surface. On the contrary, TPO2 remained in the intracellular compartment exclusively and did not reach the cell surface (Fig. 6, lanes 4 and 8).

Heme Binding and Enzymatic Activity of TPO2—Another important post-translational event for TPO is its incorporation of heme, which occurs most likely in the ER. We were interested in the consequence of the modification of the three-dimensional structure of TPO2 on such incorporation, which is required for the enzyme to function. We thus evaluated whether TPO1 and TPO2 were able to bind to the heme by adding [14C]-radiolabeled aminolevulinic acid heme precursor to cell cultures. [14C]-labeled immunoprecipitated TPO1 was detected after 16 h of labeling and markedly increased at 48 h (Fig. 7, lanes 1 and 3). In the same experiment, no [14C]-labeled TPO2 was detected, showing its inability to bind to heme (Fig. 7, lanes 2 and 4).

As TPO2 did not reach the cell surface, extracts from microsomes were used to detect peroxidase activity. Guaiacol oxidation was measured for the two TPO variants and the control pcDNA3. As expected, we detected a significant and reproducible peroxidase activity for TPO1 as compared with the negative control (Fig. 8). Conversely, TPO2 guaiacol oxidation curves remained at the same basal level of those for the negative control. This result allows us to conclude that TPO2 is enzymatically inactive.

DISCUSSION

To establish the role of TPO2 in the thyroid function, we studied the intracellular trafficking and the enzymatic activity
of this protein. We transfected the cDNA of TPO1 and of the alternatively spliced form (TPO2) in CHO cells. We generated cell lines stably expressing significant levels of each protein but observed a lower cell expression of the alternatively spliced isoform. When we immunoprecipitated the cells metabolically and observed a lower cell expression of the alternatively spliced isoform of this protein. We transfected the cells with TPO1 and with TPO2 and extracts from cells transfected with pcDNA3 were used to oxidize guaiacol. The reaction was initiated by adding H₂O₂ to reach a final concentration of 0.25 mM. Guaiacol oxidation was measured by absorbance at 470 nm and spectrophotometrically monitored every 30 s for 3 min. Variation of absorbance for each assay was calculated on the basis of a mean of three experiments.

Figure 8. Guaiacol oxidation activity of TPO1 and TPO2 isoforms. Microsomal fractions were prepared from CHO TPO cells and CHO transfected with pcDNA3 as the control. Extracts from microsomes containing the same amounts of TPO1 and TPO2 and extracts from cells transfected with pcDNA3 were used to oxidize guaiacol. The reaction was initiated by adding H₂O₂ to reach a final concentration of 0.25 mM. Guaiacol oxidation was measured by absorbance at 470 nm and spectrophotometrically monitored every 30 s for 3 min. Variation of absorbance for each assay was calculated on the basis of a mean of three experiments.

The lack of 57 amino acids in the extracellular region of TPO2 probably causes changes in the three-dimensional structure of the protein. The misfolding of TPO2 could also be potentialized by the loss of the potential site of N-glycosylation (Asn-569) in the deleted region. N-Glycans are important in ensuring the proper conformation of a protein. Alteration of a single N-linked glycosylation site may impair the protein function and lead to its degradation (35). However, it is not sure that the loss of one potential site of N-glycosylation is sufficient to totally explain the changes in immunoreactivity features of TPO2. Indeed, we reported that removal of N-glycans from TPO1 did not decrease its binding to mAbs (36), but this sugar removal occurred after the formation of a correct three-dimensional structure of the molecule.

The conformational changes in TPO2 are not sufficient to abolish the exposure of all the autoepitopes at the surface of the molecule. We demonstrated that TPO2 retains its ability to bind autoantibodies of the two patients with Hashimoto's disease. The epitopes expressed by domain A and B on the epitopic map of TPO1 have been reported to react strongly with autoantibodies, whereas epitopes from domains C and D are less involved in this recognition (25, 37). On the other hand, the linear epitope specific for mAb 47 represents less than 20% of the human autoantibody repertoire (38). However, our results suggest that at least a part of autoantibodies are expressed at the surface of TPO2. The domains recognized by the autoantibodies remain to be determined, but it will be difficult to purify enough quantities of materials because of the low quantities of TPO2 found in the cells. Note that our results on the recognition of TPO2 by autoantibodies appeared to contradict those of Finke et al. (39) who used a two-site immunoassay and recombinant TPO2 expressed in insect cells. This contradiction may result from a different folding of TPO2 in insect cells and mammal cells or from an incomplete or improper glycosylation of the protein (40). An alternative hypothesis is an aggregation of TPO2 related to its overexpression in this system. On the other hand, Tonacchera et al. (23) reported that a fragment
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from human TPO2 lacking amino acids Gly-533–Gly-590 produced by bacterial cells was still recognized by TPO autoantibodies and that the 57 spliced-out residues are probably not involved in antibody binding.

TPO is a heme-containing glycoprotein. To get more insight on the structure-function relationship of the two TPO variants, we investigated the incorporation of heme into cultured cells labeled with [14C]laminolevulinic acid. As expected, TPO1 incorporated labeled heme, and this incorporation increased with time. Heme incorporation likely occurs in the ER, as reported for myeloperoxidase precursors, which incorporated heme in the ER before acceding to final processing (41). In contrast to TPO1, TPO2 does not incorporate labeled heme despite long exposure to the isotope and an increase in TPO2 synthesized. The lack of incorporation of heme demonstrates that misfolded TPO2 does not exhibit the structural features required to bind to it. Within the 57 amino acids deleted in the TPO2 molecule, His-586 is considered as a putative proximal heme binding site.

The lack of incorporation of heme into cultured cells labeled with [14C]laminolevulinic acid. As expected, TPO1 incorporated labeled heme, and this incorporation increased with time. Heme incorporation likely occurs in the ER, as reported for myeloperoxidase precursors, which incorporated heme in the ER before acceding to final processing (41). In contrast to TPO1, TPO2 does not incorporate labeled heme despite long exposure to the isotope and an increase in TPO2 synthesized. The lack of incorporation of heme demonstrates that misfolded TPO2 does not exhibit the structural features required to bind to it. Within the 57 amino acids deleted in the TPO2 molecule, His-586 is considered as a putative proximal heme binding site.

Failure of incorporation of heme may in turn affect TPO2 trafficking in the cell. It was reported that inhibiting heme synthesis with succinyl acetone modifies the intracellular trafficking of myeloperoxidase (42). As suggested before, TPO binding to heme is expected to occur in the ER. It is not excluded that incorporation of heme is required to achieve the proper conformation of the protein that allows it to come out from the ER. That TPO2 does not bind to heme may account for the inability of TPO2 to bind to heme.

Binding of heme to TPO is obviously required for TPO to be enzymatically active. We evaluated TPO activity by measuring peroxidase activity from cell microsomes by using a guaiacol assay. As expected, non-heme-bound TPO2 exhibits no enzymatic activity. Conversely, peroxidase activity is detected in the microsomal fraction of TPO1. This is linked with the heme incorporation in the intracellular compartment where TPO is mainly found. The binding of heme to intracellular TPO1 by achieving its proper folding may thus make the protein enzymatically active. Within the thyroidocyte, these events may account for the smaller intracellular iodination of thyroglobulin (18).

In this paper, we demonstrated how normal and alternatively spliced isoforms may differ in both structure-function relation and intracellular trafficking. TPO1 enzymatic activity and immunological features have been extensively studied (1, 5, 25). Our work provides insight into the intracellular trafficking of TPO1 through the cell in relation with function and heme binding. The present data provide some advances in the knowledge of the alternatively spliced TPO2 variant. We demonstrate that TPO2 is enzymatically inactive. Although such a result was expected, the few previous studies suspected it on the basis of indirect findings (21). Furthermore, we show that the lack of enzymatic activity of TPO2 is related to the failure of incorporation of heme. This may be either the cause or the consequence of an improperly folded protein, which accounts for the changes observed in immunological reactivity to mAbs, which is related to the epitopic map of the molecule. Because of these structural changes, TPO2 is unable to reach the cell surface and remains intracellularly, where it is rapidly degraded as shown by its markedly lower half-life than that of the normal variant TPO1. Considering these structural and functional characteristics, it stands to reason that TPO2 is not implicated in thyroid hormone synthesis, but a hypothetical role for this isofrom in physiology or in thyroid diseases cannot be totally discarded.