Alternatively Spliced Form of Human Thyroperoxidase, TPOzanelli: Activity, Intracellular Trafficking, and Role in Hormonogenesis†

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ABSTRACT: Thyroperoxidase (TPO), a type I transmembrane heme containing glycoprotein, catalyzes iodide organification and thyroid hormone synthesis. One of the two main alternatively spliced forms of this enzyme, TPOzanelli, which is present in Graves's disease thyroid tissue, has a cytoplasmic domain completely modified. In the first stage of this study, the results of RT-PCR experiments showed that the TPOzanelli mRNA is present in normal thyroid tissue. We then generated CHO cell lines expressing the wild-type TPO (TPO1) and the alternatively spliced form TPOzanelli. Upon investigating a panel of 12 mAbs directed against the extracellular domain of TPO1 and sera from patients with a high titer of TPO autoantibodies, we observed that (i) the three-dimensional structure of this domain is similar in both isoforms; (ii) the autoantibodies recognize TPOzanelli as well as TPO1. The results of pulse chase and cell surface biotinylation experiments showed that the TPOzanelli has a shorter half-life (7 versus 11 h) and is expressed at the cell surface in lesser amounts than TPO1 (7 versus 15%). The total enzymatic activity and cell surface activity were determined in CHO cells expressing TPO1 and TPOzanelli, and TPO1 and TPOzanelli were found to have similar levels of activity. It was established that approximately 20% of the TPO purified from a Graves' disease thyroid gland was precipitated by polyclonal antibodies directed against a specific part of the cytoplasmic tail of TPOzanelli. This confirmed that the protein corresponding to the mRNA is present in the thyroid tissue. All in all, these results indicate that TPOzanelli can be expected to play a role in thyroid hormone synthesis and in thyroid autoimmunity.

INTRODUCTION

Thyroperoxidase (TPO)[1] is a membrane-bound hemoprotein that plays a key role 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 (T3) (*1*−*3*). TPO is also one of the main autoantigens involved in the pathogenesis of autoimmune thyroid diseases (*4*−*6*). In the presence of H_2O_2, TPO catalyzes the iodide organification of thyroglobulin at the apical membrane of the thyrocyte (*7*−*9*). The distribution and delivery of TPO are apically polarized, but less than 30% of the TPO is detected either at the thyrocyte surface (*10*, *11*) or at the surface of stable CHO cell lines expressing human TPO (*12*). Another small fraction of TPO seems to be mistargeted to the basolateral surface of thyrocytes (*10*, *13*) and may account for the hypothetical presentation of TPO to the circulating immune system, resulting in its antigenicity and its contribution to autoimmune thyroid diseases.

The human TPO (hTPO) gene codes for the full-length TPO (TPO1), a type I membrane-bound glycoprotein consisting of 933 amino acids containing a heme prosthetic group and four potential asparagine-linked glycosylation sites, with some evidence for at least one disulfide bond in the extracellular region (*14*). However, the gene generates alternatively spliced forms (for a review, see ref *5*). One alternatively spliced hTPO mRNA species has a 171 bp deletion (exon 10) and codes for a protein consisting of 876 amino acids (TPO2) (*15*−*18*). TPO2 mRNA has been found to exist in thyroid tissues from Graves' disease (*15*−*17*), as well as in normal thyroid gland (*18*). In a previous study, we established that TPO2, which is stably expressed in CHO cells, is enzymatically inactive and exhibits changes during intracellular processing and trafficking (*19*).

Another alternatively spliced hTPO mRNA has been identified by screening human thyroid cDNA libraries constructed from Graves' patient's thyroids (*20*). This mRNA species (3.2 kb) was generated by performing the alternate splicing of 130 bp which corresponds to exon 16, leading to a modified reading frame. PCR analysis showed the presence of this mRNA in three Graves' disease tissues, where it accounted for 40−50% of the total hTPO mRNAs (*20*). This

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[1] Abbreviations: TPO, thyroperoxidase; hTPO, human TPO; CHO, Chinese hamster ovary; mAb, monoclonal antibody, FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline buffer; PAGE, polyacrylamide gel electrophoresis; MMI, methylmercaptoimidazole; NHS-SS-biotin, sulfosuccinimidyl)2-(biotinamido) ethyl-1, 3-dithiopropionate; ER, endoplasmic reticulum; BSA, bovine serum albumin.

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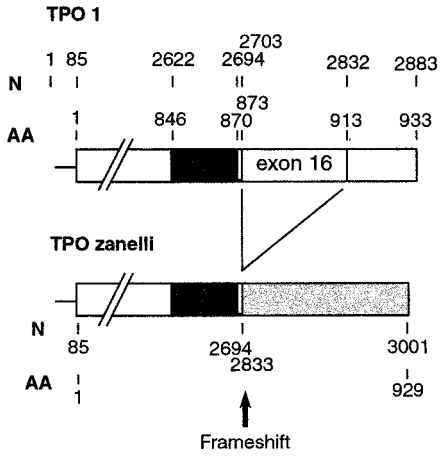

FIGURE 1: Alignment of nucleotide (N) and amino acid (AA) sequences of TPO1 and TPOzanelli. In TPOzanelli, the alternative splicing of exon 16 leads to the translation of exon 17 immediatly after exon 15 in a modified reading frame. Transmembrane domains are shown in black.

mRNA species encodes a 929 amino acid protein (named TPOzanelli) (*6*) with 56 modified-COOH terminal amino acids (see Figure 1). Note that 10 years after the discovery of TPOzanelli specific mRNA, nothing further is known about the protein, its enzymatic and immunological activities, or the part it may possibly play in thyroid physiology and pathology.

The aim of the present study was therefore (i) to search for the presence of both TPOzanelli mRNA and protein in thyroid tissue; (ii) to study the structural, functional, and immunological aspects of TPOzanelli using stable CHO cell lines expressing TPOzanelli. We used a structural approach to the molecule, consisting of mapping its antigenic surface, using a panel of TPO mAbs to compare it with the major protein TPO1. We then studied both the localization and the intracellular trafficking of the protein in CHO cells. It was also proposed to determine whether TPOzanelli was enzymatically active. To gain further insights into the role played by this variant in thyroid physiopathology, we tested its immunoreactivity against serum autoantibodies from patients with autoimmune thyroid diseases, and established that it is present in thyroid tissue.

EXPERIMENTAL PROCEDURES

RNA Isolation from Various Thyroid Tissues. We used frozen thyroid tissues from patients with normal thyroid tissue and a solitary adenoma. Tissues were homogenized and prepared using the RNeasy total RNA kit (Quiagen, Chatsworth, CA) according to the manufacturer's instructions. After determining RNA concentration of each sample spectrophotometrically from the absorption at 260 nm, they were aliquoted at a concentration of 1 mg/mL and stored in water at −80 °C until further use. The absorption ratio (260/280) was between 1.7 and 1.9 with all the preparations, and the integrity of the RNA was checked on agarose gel colored with ethidium bromide.

Reverse Trancription (RT) and Polymerase Chain Reaction (PCR) Amplification. With each mRNA, a specific first-strand synthesis was performed using 1 μg of total RNA added to 200 UI of Moloney Murine Leukemia Virus reverse transcriptase (M-MLV ReverseTranscriptase, Gibco BRL, Life Technologies, Cergy Pontoise, France) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.2 mM deoxynucleotide triphosphate, and 100 pmol oligo pd(N)6 (Pharmacia Biotech, Upsala, Sweden), in a final volume of 20 μL. Samples were incubated for 1 h at 37 °C, and the reverse transcriptase was then inhibited by incubating for 5 min at 95 °C.

Twenty microliters of the RT reaction medium was added to 80 μL of PCR mixture (10 mM Tris-HCl, pH 8.3, 100 mM KCl, 0.75 mM EGTA, 5% glycerol, and 1.5 mM $MgCl_2$) containing 0.2 mM deoxynucleotides triphosphates, 5 units of Taq polymerase (Gibco BRL, Life Technologies, Cergy Pontoise, France) and 15 pmol each of the sense and antisense primers: FP, 5′-CTGCGCGGACCCCTACGAGTTAGG, and RP, 5′-GGAAAAGAGTCGTACGGTGATTTTGGG. The microtubes were then overlaid with mineral oil and kept in ice until the thermal cycler was up to 94 °C. The samples were subjected to 30 PCR amplification cycles including denaturation for 30 s at 95 °C, hybridation for 90 s at 59 °C, and elongation for 90 s at 72 °C. Twenty microliters of each of the amplification products were analyzed on 2% agarose gel stained with ethidium bromide. Amplification of pcDNA3-TPOzanelli was used as a positive control in each of the PCR reactions.

Construction of pcDNA3-TPOzanelli. Full-length 3060 kb TPO1 cDNA kindly provided by B. Rapoport was cloned into the *Hin*dIII and *Xba*I sites of the eukaryotic transfer vector pcDNA3 (Invitrogen, Leak, The Netherlands). The 869 bp insert of a cDNA clone was isolated from a human Graves' disease thyroid λgt11 cDNA library (*20*). This 3′ TPOzanelli insert corresponds to the 3′ end of the alternatively spliced TPOzanelli with the 130 bp deletion of exon 16 and contains at both extremities the *Eco*RI restriction, and was previously cloned into pUC18 (Pharmacia Biotech, Upsala, Sweden). The 5′ end (1−2600 bp) of the hTPO1 cDNA was released from TPO1-pcDNA3 construction by digestion using the *Acc*I restriction enzyme, as in the case of the pUC18-3′ TPOzanelli insert. In this insert, the *Acc*I restriction site was located 98 bp upstream of the junction region due to the deletion of 130 bp, and thus contained the specific nucleotide shift frame of the alternatively spliced variant. The 2600 bp from the TPO1 cDNA fragment were then replaced upstream of the 3′ end of insert to recover the full-length TPOzanelli cDNA, as confirmed by sequence analysis. The TPOzanelli cDNA was then removed from the TPOzanelli-pUC18 construct and replaced into the *Hin*dIII and *Eco*RI sites of the eukaryotic transfer vector pcDNA3. The *E. coli* strain XL1 Blues (Stratagene, La Jolla, CA) was transformed with the TPOzanelli-pcDNA3 construct and pure plasmid DNA preparations were obtained with the Wizard midipreps kit (Promega, Madison, WI). Enzyme reactions and DNA manipulations were carried out as described in Maniatis et al. (*21*).

CHO Cell Cultures and Transfection. CHO cells (ECACC no. 85050302) were maintained in Ham's F12 medium supplemented with 10% FBS, penicillin (100 UI/mL) and streptomycin (0.1 mg/mL). Cells were transfected by the lipofectamine method (Gibco-BRL, Life technologies, Cergy-Pontoise, France) with either pcDNA3-TPO1 or pcDNA3-TPOzanelli, or pcDNA3 alone as control. Cells were incubated in a saturated atmosphere (5% CO_2−95% air) at 37 °C. Stable transfectants were selected in the presence of

Geneticin (400 μg/mL) and were subcloned using limiting dilutions. Positive TPO expressing cell lines were identified by performing Westernblotting or immunoprecipitation after [^{35}S](Met + Cys) labeling (Expre ^{35}S ^{35}S Protein Labeling Mix, Dupont NEN, le Blanc Menil, France). A significant level of TPO1 and TPOzanelli expression was obtained by culturing CHO−TPO cell lines with 10 mM sodium butyrate, as previously reported (*12*, *19*).

Metabolic Labeling of TPO. Cells were incubated in cysteine and methionine-free DMEM supplemented with 10% FBS, 10 mM sodium butyrate, and 100 μCi/mL of [^{35}S]-(Met + Cys). Incubation was carried out for 5 or 48 h. In the pulse−chase experiments, cells were incubated for 1 h in Cys and Met-free DMEM supplemented with 10% dialyzed FBS and 10 mM sodium butyrate. Cells were then pulsed for 30 min in the presence of 100 μCi/mL [^{35}S](Met + Cys). After the pulse, the labeling medium was removed and the cell surface was washed three times with PBS and then replaced by DMEM supplemented with 10% FBS, 5 mM Met and 5 mM Cys. Chases were performed for 30 min, 1, 5, 22, 48, and 72 h.

Cell Lysis and Immunoprecipitation. After being metabolically labeled, cells were harvested on ice by scraping them into 1 mL of PBS and centrifuged at 700*g* for 7 min. Cell pellets were resuspended in 600 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, Germany)], vortexed every 2 for 20 min, and centrifuged at 10 000*g* for 5 min. Radiolabeled supernatants were incubated for 2 h at room temperature with a couple of mAbs recognizing either a sequential region (mAb 47) or a conformational epitope (mAb 15) of the TPO molecule (*22*). mAbs were chosen on the basis of their strong recognition of each isoform in a panel of TPO-mAbs. These mAbs were previously complexed with protein-A Sepharose 4B (Zymed Laboratories, San Francisco, CA) by incubation overnight at 4 °C. Immune complexes were then retrieved by a brief centrifugation (10000*g*, 10 s) and washed six 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 them for 5 min in 80 mL of electrophoresis buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 5% glycerol) and then analyzed by SDS−PAGE (7.5%). Protein associated radioactivity was visualized and quantified by a phosphorimager (Fudjix BAS 1000).

TPO was also immunoprecipitated with a panel of mAbs directed against various antigenic domains of the TPO1 molecule (*22*). In this experiment, [^{35}S](Met + Cys) radiolabeled CHO-TPO cell lysates were immunoprecipitated for 4 h at 25 °C with 50 μg of each of the TPO-mAbs previously complexed with protein-A Sepharose 4B.

Cell Surface Biotinylation. TPO1-CHO and TPOzanelli-CHO confluent monolayers were metabolically labeled for 18 h with 100 μCi/mL [^{35}S](Met + Cys) in the presence of 10 mM sodium butyrate, and cell surface biotinylated as described in refs *12* and *24*. Cells were washed twice with PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and exposed to a 0.5 mg/mL Immunopure NHS-SS-Biotin (Pierce, Oud Beijerlank, The Netherlands) for 20 min at 4 °C. The cross-linker was removed and the same procedure repeated once. The biotin reagent was quenched by incubating the preparation with 50 mM NH_4Cl 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 10% SDS, boiled for 5 min, diluted with 600 μL of TPO-extraction buffer and centrifuged (10000*g*, 3 min). Supernatant containing total TPO was incubated 2 h with avidin-agarose (Pierce, Oud Beijerlank, The Netherlands). Biotinylated surface TPO and intracellular TPO were separated by centrifugation (10000*g*, 10 s). The beads were washed four times with TPO-extraction buffer and once with PBS, resuspended in electropheresis buffer and boiled for 5 min. The supernatants were analyzed by SDS−PAGE.

TPOzanelli Total Enzymatic Activity. Microsomal fraction pellets, prepared as described in ref *19*, were solubilized by resuspending them in 15 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1 mM KI. Microsomes prepared from CHO cells transfected with pcDNA3 were used as a negative control. Microsomal fractions were centrifuged (10000*g*, 2 min), and the supernatant was used for the enzymatic assay. Extracts containing approximately the same amounts of TPO1 and TPOzanelli were added to 1 mL of 40 mM guaiacol (Fluka Chimie, St. Quentin-Fallavier, France) and 67 mM sodium phosphate buffer, pH 7.5. The reaction was performed at room temperature and initiated by adding H_2O_2 to reach a final concentration of 0.25 mM. Guaiacol oxidation was measured by absorbance at 470 nm and monitored spectrophotometrically every 30 s for 3 min.

TPOzanelli Cell Surface Enzymatic Activity. Cell surface enzymatic activity was assayed as in ref *25*, with slight modifications. TPOzanelli-CHO cells were incubated in Ham's F12 medium supplemented with 10 mM sodium butyrate for 18 h. CHO cells transfected with pcDNA3 alone or TPO1-CHO cells were used as a negative or positive control, respectively. The medium was removed and cells washed twice with PBS before being incubated with BSA (5 mg/mL in PBS) and $Na^{125}I$ (10^6 cpm/mL), with or without 2 mM 2-mercapto-1-methylimidazole (MMI), as the control medium. The reaction was initiated by adding H_2O_2 to obtain a final concentration of 0.5 mM, and cells were incubated for 20 min at room temperature. The medium was then transferred to cold reaction tubes; the cell surface was washed out with 0.5 mL of PBS and the wash was added to the medium. Each tube was filled with 1 mL of ice-cold 20% (wt/vol) trichloroacetic acid, supplemented with 10^{-4} M KI and was incubated for 20 min at 4 °C, before being centrifuged (2000*g*, 6 min). The supernatant was discarded, and the acid-insoluble iodinated material obtained was washed three times with 2 mL of 10% (w/v) trichloroacetic acid. The radioactivity remaining in the pellet was counted.

Analysis of TPOzanelli Immunoreactivity to TPO-Autoantibodies. TPOzanelli-CHO cell lysates were also tested against TPO autoantibodies present in the sera from four patients with Haschimoto's thyroiditis or Graves' disease. Two had TPO-autoantibody titers of between 100 and 1000 UI/mL and two others had titers greater than 10 000 UI/mL, as measured by a two-site chemoluminescent assay (Lumitest anti-TPO, Brahms, Berlin, Germany). Each serum (500 μL) was complexed with protein-A Sepharose 4B by overnight incubation at 4 °C. Serum from a normal individual with no detected thyroid autoantibodies was used as the control serum. Metabolically labeled TPOzanelli was ex-

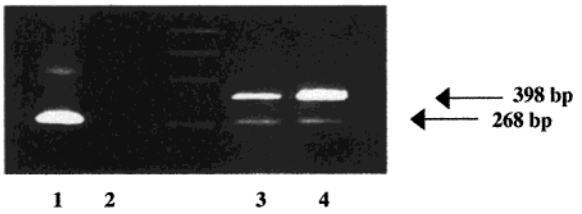

FIGURE 2: Reverse transcription and PCR amplification products of TPOzanelli mRNA variant. RNA was isolated from normal (lane 3) and adenomatous thyroid tissue (lane 4). Tissues were homogenized and prepared using the RNeasy total RNA kit, and reverse trancription (RT) and polymerase chain reaction (PCR) amplification were then performed as described in Materials and Methods. The samples were subjected to 30 cycles of PCR amplification including denaturation for 30 s at 95 °C, hybridation for 90 s at 59 °C, and elongation for 90 s at 72 °C. Amplification of pcDNA3-TPOzanelli (lane 1) was used as a positive control, along with the negative control (lane 2) in each of the PCR reactions. Amplification products were analyzed on 2% agarose gel stained with ethidium bromide.

tracted as previously described and then incubated for 2 h at room temperature with autoantibody/protein A-Sepharose 4B complexes. These complexes were retrieved by performing a brief centrifugation and treated in the same way as the mAbs. TPOzanelli immunoprecipitated by autoantibodies was eluted from the complexes and analyzed by performing SDS−PAGE.

Presence of TPOzanelli in Human Thyroid Cells. Antipeptides antibodies directed against the intracytoplasmic part of the TPOzanelli were obtained by immunizing female New Zealand rabbits with peptide (amino acid 904−914)/KLH conjugated emulsified in complete Freud's adjuvant. The animals were given a booster injection 5 weeks later. Sera were tested to determine their antibody titer against the peptide by ELISA. Human hTPO was extracted from fresh Graves' thyroid, purified by affinity chromatography using mAb 15 as described previously (*4*), and then 125I radiolabeled. This labeling was carried out by incubating 2.5 μg of purified hTPO with 0.2 mCi 125I Na in 15 μL of PBS. The reaction was initiated by adding H_2O_2 (0.3 mM final). H_2O_2 was then added after 1 and 2 min of incubation (in order to obtain final amounts of 1 mM and 3 mM H_2O_2, respectively). [^{125}I]hTPO was purified using a PD10 column (Amersham-Pharmacia). [^{125}I]hTPO was immunoprecipitated either by the couple mAb15 + mAb 47, by anti TPOzanelli antipeptide, or by nonimmunized rabbit serum as the control serum, as described above.

RESULTS

Expression of TPOzanelli mRNA in Thyroid Tissues. Total RNA was extracted from each tissue sample and reverse transcribed. PCR was then performed using the primers annealing sequence within exon 14 and 17. PCR amplification was also performed using the TPO1-pcDNA3 and TPOzanelli-pcDNA3 constructs as templates, which were used as positive controls (Figure 2). Two migrating bands with an apparent size of 398 bp and 268 bp were observed, corresponding to TPO1 and exon 16-deleted TPOzanelli, respectively, as compared to the positive control. Both bands were observed in normal tissue as well as benign adenoma. This result shows that TPOzanelli mRNA is present in normal thyroid tissues as well as in thyroid tissue from Grave's disease (*20*) and benign adenoma.

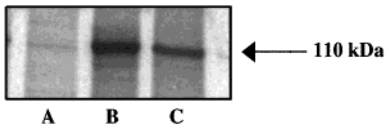

FIGURE 3: Immunoprecipitation of [^{35}S] (Met + Cys) labeled TPO1 and TPOzanelli from stably transfected CHO cell lines. Cells were incubated with [^{35}S] (Met + Cys) for 48h and then lysed. TPO1 and TPOzanelli were immunoprecipitated with mAb 47 + mAb 15 for TPO1 (lane B) and TPOzanelli (lane C). CHO cells transfected with the eucaryotic vector pcDNA3 were used as controls (lane A). Samples were run on 7.5% SDS−polyacrylamide gels and the bands were detected using a phosphorimager.

Expression of TPOzanelli in CHO Cell Lines. CHO cells were transfected with human TPOzanelli cDNA and isolated cell clones expressing significant levels of the TPOzanelli variant were isolated. Expression of the protein was evaluated by immunoprecipitating TPO1 and TPOzanelli with a combination of two TPO-mAbs, after performing metabolic labeling of TPO1- and TPOzanelli-CHO cells with [^{35}S](Met + Cys). As was to be expected, TPO1 gave a 110 kDa specific band (Figure 3, lane B) and TPOzanelli showed up as a band with the similar apparent size on the SDS−PAGE analysis (Figure 3, lane C). These two bands were shown to specifically reflect TPO1 and TPOzanelli isoforms, and were not present in CHO cells transfected with pcDNA3 alone (Figure 3, lane A).

Structural Characteristics of TPOzanelli. Alternative splicing of exon 16 leads to the translation of exon 17 immediately after exon 15 and generates a shift in the TPOzanelli gene reading frame. TPOzanelli mRNA thus encodes a 929 amino acids protein with a modified 56-COOH terminal amino acid sequence. The hydropathy profile has been reported to predict an elongated membrane-spanning domain consisting of 40 residues (13 amino acids longer than that of TPO1), and a 43 amino acid cytoplasmic region (*20*). However, sequence analysis using two different transmembrane domains research programs (*26*, *27*) shows no difference in the length of the predicted transmembrane domain of TPO1 and TPOzanelli (data not shown) suggesting that the TPOzanelli would differ from the TPO1 only by its cytoplasmic tail (see Figure 1). Although the extracellular part of the protein remains unchanged, the changes undergone by the cytoplasmic part might affect the overall three-dimensional structure. To determine whether TPOzanelli shows any three-dimensional changes in comparison with TPO1, we determined its immunological reactivity against a panel of mAbs directed to TPO1 (*22*). Only the mAb 47 recognizes a sequential epitope on the TPO molecule, the others are directed against conformational epitopes, as previously reported (*22*, *23*). After undergoing [^{35}S](Met + Cys) metabolic labeling for 16 h, TPO1 and TPOzanelli were tested by immunoprecipitation with each of the 12 TPO mAbs. TPO1 and TPOzanelli immunoreactivity were observed with all the mAbs tested (Figure 4, panels A and B), particularly against mAb 47 and mAb 15, which led us to use these mAbs in both immunoprecipitation experiments on TPO1 and TPOzanelli. Three out of 12 mAbs (mAbs 53, 59, and 64) had a lower reactivity to TPOzanelli than to TPO1 (Figure 4C).

TPOzanelli Immunoreactivity against Thyroid Autoantibodies. We determined the antigenic immunoreactivity of TPOzanelli against sera from patients with high titers of TPO

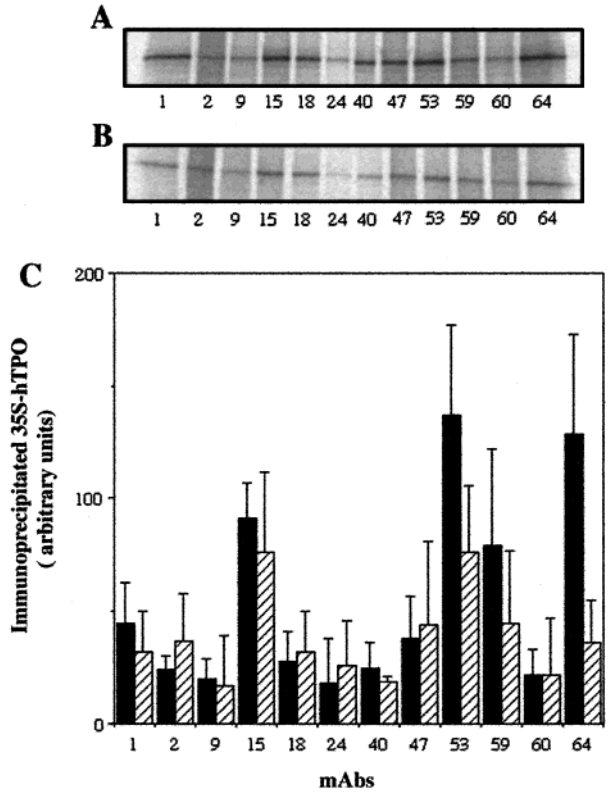

FIGURE 4: Analysis of TPO1 and TPOzanelli immunoreactivity against TPO-mAbs. TPO1 (A) and TPOzanelli (B) were immunoprecipitated with a panel of mAbs directed against various antigenic domains of the TPO1 molecule (*22*). TPO-CHO cells were incubated with [^{35}S] (Met + Cys) for 16 h. Immunoprecipitated TPO was recovered from mAb-protein A-Sepharose 4B complexes and then analyzed by SDS−PAGE (7.5%). Protein associated radioactivity was detected and quantified by phosphorimaging (C) with TPO1 (black) and TPOzanelli (hatched).

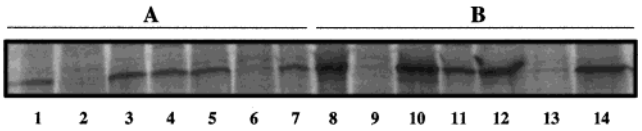

FIGURE 5: Analysis of TPO1 (A) and TPOzanelli (B) immunoreactivity against TPO autoantibodies. TPOzanelli was tested against TPO autoantibodies in the sera from two patients with Haschimoto's thyroiditis (lanes 3, 4, 10, and 11) and two patients with Graves' disease (lanes 5, 7, 12, and 14). Serum from a normal individual (lanes 2 and 9), TPOzanelli immunoprecipitated with mAb47 + mAb15 (lanes 1 and 8), and CHO cells transfected with the eucaryotic vector pcDNA3 tested with serum from one patient with Haschimoto's thyroiditis (lanes 6 and 13) were used as controls. Radiolabeled TPOzanelli immunoprecipitated by autoantibodies was eluted from complexes and analyzed by 7.5% SDS−PAGE.

autoantibodies. The results of immunoprecipitation experiments showed that serum containing TPO autoantibodies bound specifically to TPOzanelli (Figure 5, lanes 10, 11, 12, and 14), whereas the serum of normal individuals did not (Figure 5, lane 9). The band intensities which were determined on account of the non specific background, showed a similar pattern than those observed with TPO1 (Figure 5, lanes 3, 4, 5, and 7). These similarities suggest that serum thyroid autoantibodies recognize TPOzanelli as well as TPO1.

Stability, Localization, and Intracellular Trafficking of TPOzanelli in CHO Cells. To investigate whether the structural changes in TPOzanelli alter the stability of the

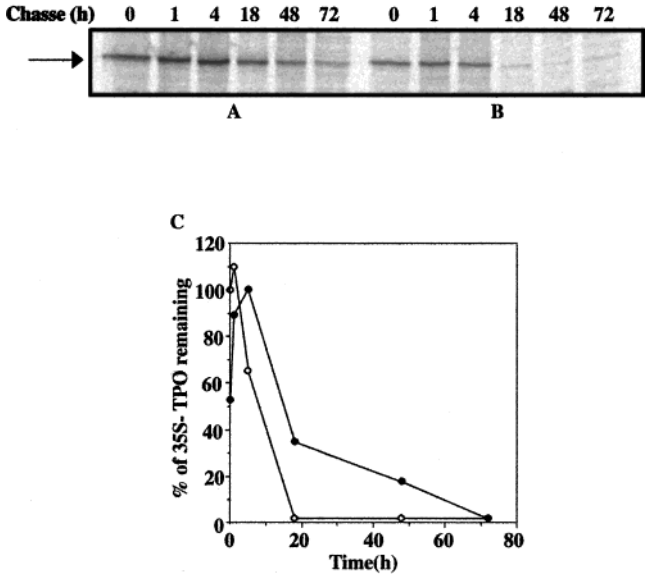

FIGURE 6: Rate of degradation of TPO1 and TPOzanelli. TPO-CHO cells were pulsed for 30 min in the presence of 100 μCi/mL [^{35}S](Met + Cys). After the pulse procedure, the labeling medium was removed and replaced by DMEM supplemented with unlabeled amino acids. At the times indicated, radiolabeled cell lysates were immunoprecipitated with mAb47 + mAb15 for TPO1 (A) and TPOzanelli (B). Immunoprecipitated TPO was analyzed by SDS−PAGE and detected by phosphorimaging. The arrows indicate TPO1 or TPOzanelli bands identified in each experiment. Each labeled band of TPO1 (●) and TPOzanelli (○) was quantitated by phosphorimaging (C).

glycoprotein, we performed pulse-chase studies and determined the half-life of TPO1 and TPOzanelli. Cells were pulsed for 30 min in the presence of [^{35}S](Met + Cyst), and chased at various times. Immunoprecipitated TPO1 and TPOzanelli were analyzed by SDS−PAGE (Figure 6). [^{35}S]-(Met + Cys) TPO1 was still detected after 72 h of chase (Figure 6A). By contrast, radiolabeled TPOzanelli was not detected after 18h of chase (Figure 6B), which suggests that TPOzanelli is degraded more rapidly than the major variant in the cell. Phosphorimager quantification of both TPO1 and TPOzanelli in several experiments showed that TPOzanelli (7 h) had a shorter half-life than TPO1 (11 h) (Figure 6C).

We then investigated whether the structural changes in the TPOzanelli variant might affect the localization and the intracellular trafficking of the molecule. We therefore evaluated the cell surface expression of the TPO1 and TPOzanelli isoforms by performing surface biotinylation experiments. Cells were [^{35}S](Met + Cys) metabolically labeled in the steady state (48 h), and the surface proteins were tagged by biotinylation with NHS−SS-biotin, a reagent that reacts with the amino groups of extracytoplasmic lysine residues. TPO was recovered by immunoprecipitation, released from the mAb by denaturation in SDS, and incubated with avidin-agarose. This procedure separated the biotinylated surface TPO bound to avidin-agarose from the intracellular TPO, which was inaccessible to the tagging agent. Like TPO1, TPOzanelli is mainly to be found in the intracellular compartments (Figure 7, panels B and C). However, lower amounts of TPOzanelli (7%) (Figure 7, panels B and C) than of TPO1 (15%) are present at the cell surface (Figure 7, panels A and C).

Enzymatic Activity of TPOzanelli. The total peroxidase activity was determined by measuring the guaiacol oxidation

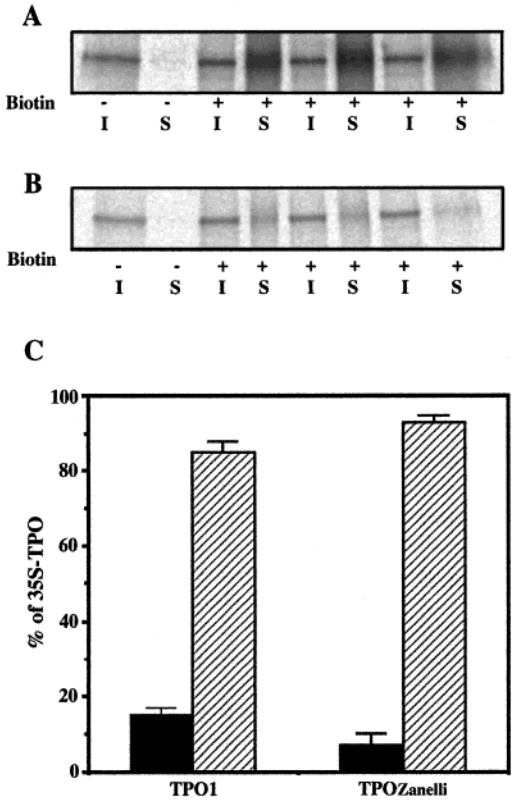

FIGURE 7: Distribution of TPO1 and TPOzanelli in CHO cells. Cells were metabolically labeled for 48 h with [^{35}S](Met + Cys). Cell surface biotinylation was performed as described in Materials and Methods. TPO1 (A) and TPOzanelli (B) were immunoprecipitated as described, from cell lysates and then reprecipitated with avidin-agarose. All the supernatant corresponding to the cell surface (S in panels A and B, black in panel C) tagged fraction (avidin-bound), and only one tenth of the supernatant corresponding to the intracellular (I in panels A and B, hatched in panel C) fraction (avidin-unbound) were analyzed by 7.5% SDS−PAGE. Radiolabeled TPO1 and TPOzanelli (I and S) were detected and quantified by a phosphoimager. Data were established on the basis of the mean data from three experiments; experiments were carried out with (+) and without NHS-SS-biotin reagent (−).

process in microsomal preparations. As expected, peroxidase activity was detected in the case of TPO1. TPOzanelli also showed a significant and reproducible level of enzymatic activity as compared to the negative control (Figure 8).

The cell surface enzymatic activity was also determined from the ability of TPO to catalyze the radioiodination of BSA at the surface of the CHO cells. As expected, a significant and reproducible peroxidase cell surface activity was detected in the case of TPO1 as compared to the negative control (Figure 9), and this activity decreased upon adding MMI. TPOzanelli also exhibits a detectable level of enzymatic activity. This enzymatic activity is 2.5 times lower than that obtained with TPO1, which is quite normal in view of the quantitative difference between the TPO1 and TPOzanelli present at the cell surface (15 versus 7%)

Presence of TPOzanelli in Normal Thyroid Tissue. To determine whether TPOzanelli is present in normal human thyroid tissue, hTPO was purified from Graves' disease thyroid glands by performing affinity chromatography using mAb 15 and then radiolabeled using [^{125}I]Na. After immunoprecipitation using rabbit polyclonal antibodies directed against a peptide specifically recognizing TPOzanelli, [^{125}I]-TPOzanelli (Figure 10, lanes 3 and 4) was recovered as a

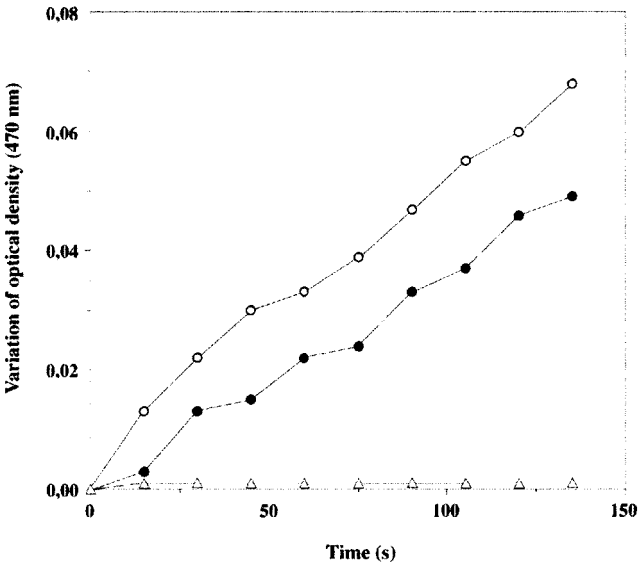

FIGURE 8: Guaiacol oxidation activity of TPO1 and TPOzanelli isoforms. Microsomal fractions were prepared from TPO1- and TPOzanelli-CHO cells and CHO cells transfected with pcDNA3 as controls. Extracts from microsomes containing the same amount of TPO1 (○) and TPOzanelli (●) and extracts from cells transfected with pcDNA3 (△) were used to oxidize guaiacol. The reaction was initiated by adding H_2O_2 to obtain a final concentration of 0.25 mM. Guaiacol oxidation was measured by absorption at 470 nm and monitored spectrophotometrically every 15 s.

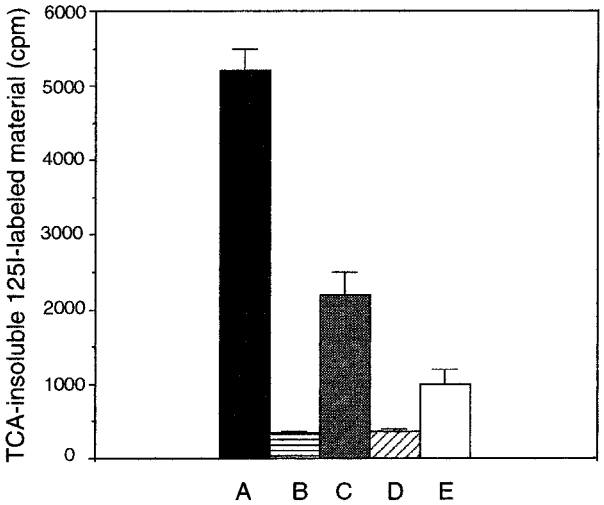

FIGURE 9: Enzymatic activity of TPO1 and TPOzanelli at the cell surface of transfected CHO cells. TPO1- (A), TPOzanelli-CHO cells (C), or CHO cells transfected with pcDNA3 alone (E) were preincubated 18 h with 10 mM sodium butyrate. The incubation mixture contained BSA (5 mg/mL in PBS) and [^{125}I]Na (10^6 cpm/mL). Negative controls in which 2 mM mercapto-1-imidasole was added were also tested with TPO1- (B) and TPOzanelli (D). The reaction was initiated by adding H_2O_2 to a final concentration of 0.5 mM. Cells were incubated for 20 min at room temperature, transferred to reaction tubes and precipitated with 1 mL 20% trichloracetic acid for 20 min at 4 °C, then centrifuged. The supernatant was discarded, and the acid-insoluble iodinated material obtained was washed three times with 2 mL of 10% trichloracetic acid. The radioactivity remaining in the pellet was counted. The data were based on three different experiments.

part (approximately 20%) of the total [^{125}I]hTPO, which shows that the TPOzanelli isoform is present in the normal human thyroid. The total [^{125}I]TPO immunoprecipitated by the couple of anti-TPO mAbs 47 and mAb 15 (Figure 10,

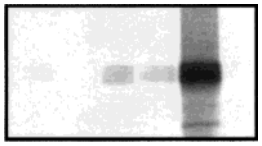

FIGURE 10: Immunoprecipitation of the TPOzanelli isoform with specific antipeptide antibody. Human TPO was extracted from human thyroids, ^{125}I-labeled, then immunoprecipitated with rabbit polyclonal antipeptide antibody directed to the specific intracytoplasmic domain of human TPOzanelli (lanes 3 and 4). Sera of nonimmunized rabbits (lanes 1 and 2) and anti-TPO mAbs (15 + 47) (lane 5) were used as controls.

lane 5) and by nonimmune rabbit sera (Figure 10, lanes 1 and 2) were used as controls.

DISCUSSION

Although the alternatively spliced TPOzanelli mRNA has been found to exist in Graves' thyroid glands (*20*), no further information has been published about the structural and functional characteristics of the protein. These questions were therefore investigated here by performing transfection experiments using TPO1 and TPOzanelli cDNAs in CHO cells and by generating stable cell lines expressing significant levels of each protein.

To investigate some of the structural aspects of TPOzanelli, we used a structural approach consisting of mapping the antigenic surface, using a panel of 12 mAbs directed against TPO1 (*22*). Three mAbs which are directed against conformational epitopes misrecognize TPOzanelli, which suggests that their epitopes are poorly exposed. However, most of the TPO1 conformational epitopes were recovered at the surface of TPOzanelli. The decrease in the recognition of TPOzanelli by mAb 59 may be attributable to its low affinity, whereas mAb 64 has been reported to be sensitive to minor structural changes in the TPO molecule, such as coating to a solid phase in binding experiments (*22*). These data suggest that only slight changes in the three-dimensional structure of the molecule have occurred in comparison with TPO1.

TPO is also the main autoantigen involved in thyroid disease. Here we established by performing immunoprecipitation experiments that sera from patients with Hashimoto's thyroiditis and Graves' disease recognize TPOzanelli. Our results agree with those obtained by Finke and al, using a two-site immunoassay and recombinant TPOzanelli expressed in insect cells (*28*). TPOzanelli was actually expected to be involved in autoantigenicity, as the extracellular domain of TPOzanelli has remained unchanged in comparison with TPO. However, two mAbs (mAb 59 and 64), which recognized TPOzanelli much less strongly than TPO1 are directed against epitopes expressed by domain B on the epitopic map of TPO1, and have been reported to react strongly with autoantibodies, whereas epitopes from domains C and D are less involved in this recognition process (*22*). The fact that serum autoantibodies strongly recognize TPOzanelli suggests that TPOzanelli auto-antigenicity may depend on the exposure to other auto-epitopes. The exact domain(s) recognized by the autoantibodies still remain to be determined and may be restricted to pathological conditions.

To determine whether the change in the COOH terminal amino acid sequence has any effect on the stability and the intracellular trafficking of the protein, we studied the stability of TPOzanelli in pulse-chase experiments from cells metabolically labeled with [^{35}S](Met + Cys). As compared to TPO1, the stability of the protein was impaired, as no labeled TPOzanelli was detected in the cell for periods of up to 18 h. Structural changes within a molecule have been reported to alter the stability of a protein, as in the case of the other spliced form, TPO2 (*19*).

A shorter half-life was also associated with changes in the intracellular trafficking of the TPOzanelli variant occurring in CHO cells. Cell surface biotinylation experiments showed that only 7% of the protein are present at steady state at the cell surface, as compared to 15% in the case of TPO1. Both TPO isoforms are mainly located at the intracellular level within the cell. This mainly intracellular localization of TPO1 has been described in the case of CHO cells (*13*), as well as in thyrocytes, where no more than 30% of the total TPO1 was detected in the plasma membrane (*10*). In the steady state, a lower fraction of TPOzanelli than of TPO1 reaches the cell surface. This situation suggests that TPOzanelli may be more retained in the cell. It has been reported that differences in the processing of a protein may decrease the trafficking to the cell surface, and that incorrectly folded proteins are retained mainly in the ER (*29*−*31*). In the case of TPO1, we previously established that only 2% of the synthesized protein can reach the cell surface (*19*) and that the remainder is degraded at the ER level by two different processes, depending on the folding of the protein (*32*). The TPOzanelli variant differs from TPO1 in the deletion of 130 bp and the modified-COOH terminal domain due to the shift in the reading frame and the use of a different stop codon. The present mapping analysis showed that only minor structural changes may have occurred in the extracellular part of the molecule. Sequence analysis of the modified cytoplasmic domain showed that it lacks a diacidic motif D-X-E encoded by nucleotides present within the spliced exon 16. This specific diacidic motif present in the cytoplasmic tail of membrane protein has been reported to serve as the sorting signal required by a protein to be able to leave the ER (*33*). The lack of this sorting signal may be responsible for the lower ability of TPOzanelli to be exported from the RE to the cell surface.

We observed here that TPOzanelli is enzymatically active, since we measured its ability to oxidize guaiacol in the presence of H_2O_2. In addition, we established that TPOzanelli is enzymatically active at the cell surface. TPOzanelli enzymatic activity, expressed as the ratio between the TPO-CHO and wild-type CHO activities, turned out to be similar to that observed in the case of TPO1. TPOzanelli was expected to be functional since the extracellular domain of the molecule remains unchanged and the critical residues (i.e., the histidines involved in the binding to heme and N-glycosylation sites) are still present (*34*, *35*). In addition, the modified COOH terminal domain did not appear to disrupt the three-dimensional structure of the molecule required for its functional activity. TPOzanelli is therefore enzymatically active, and can reach the cell surface.

Since TPOzanelli mRNA was found to be present in Graves' disease thyroid glands (*20*), no further data have been published on the presence of the TPOzanelli protein in thyroid gland. We established here that TPOzanelli mRNA is present in normal thyroid glands and thyroid follicular

adenoma, although we were not able to establish the exact levels or the ratio between the two TPO mRNAs. Alternate splicing may thus be involved in the control of TPO gene expression independently of the cellular transformation processes. It was furthermore established here that the protein TPOzanelli is also present in thyroid cells, forming part of the total pool of hTPO present in human thyroid cells.

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