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Molecular Analysis of Mutated Thyroid Peroxidase Detected in Patients with Total Iodide Organification Defects*

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ABSTRACT

Wild-type and mutant thyroid peroxidase (TPO) was expressed in a Semliki Forest Virus (SFV)-based transient expression system in Chinese hamster ovary-K1 cells. Twenty four hours after transfection, proteins immunoreactive with TPO antibodies could be detected on a Western blot. Peroxidase activity was assayed using both the guaiacol and the I_3^- assay. Addition of hematin was necessary to obtain enzymatic active TPO. Thyroid peroxidase complementary DNA constructs containing mutations originally found in patients with hereditary congenital hypothyroidism caused by total iodide

organification defects were analyzed using these techniques. In all cases TPO was expressed as shown by Western blotting and immunostaining. Enzymatic activity (measured by guaiacol and iodide oxidation assay) was below the detection level in four out of five mutants. The only mutant yielding TPO with enzymatic activity was G 1858 A (Gly 590 Ser). However, the mutation could affect splicing of TPO messenger RNA, leading to inactive TPO, because it is located at the exon 10/intron 10 border. (*J Clin Endocrinol Metab* 82: 649–653, 1997)

THYROID PEROXIDASE (TPO) is a membrane-linked hemoprotein located at the apical membrane of the thyroid cell. The major part of the enzyme containing the heme group is facing the luminal colloid of the thyroid cell. TPO catalyzes the iodination and subsequent coupling of tyrosine residues in thyroglobulin, resulting in the synthesis of the thyroid hormones T_4 and T_3 (1, 2).

Generation of recombinant human TPO displaying immunological and enzymatic activity has been reported (3–5). Also, a secreted form of TPO lacking the transmembrane domain was stably expressed in Chinese hamster ovary (CHO) cells (6).

Defects in the function of TPO result in total iodide organification defects (TIODs). Complete absence of thyroid peroxidase activity has been described in patients with TIODs (7–9).

We were able to investigate thyroid tissue from three unrelated TIOD patients (9–11), and TPO messenger RNA (mRNA) levels varied between not detectable (9) and elevated (11). In thyroid tissue of the third patient, the TPO-1 mRNA level was very low because of an early termination codon, whereas alternatively spliced TPO-2 mRNA was present (10). These three patients carried homozygous mutations in exons 2, 9, and 10, respectively (Table 1, mutation numbers 1, 4, and 5).

As a result of early detection and treatment of TIOD patients, the development of goiter is rare. As a consequence,

thyroid tissue from these patients is seldom obtained. For diagnosis at the molecular level, genomic DNA was screened for mutations in the TPO genes of TIOD patients (11). From about 40 Dutch families in which it is known that TIOD occurs, 13 unrelated families were investigated (Table 1). All patients but one showed mutations in both TPO alleles. We identified eight different mutations in 25 alleles in which the TPO gene cosegregated with the iodide organification defect (11). From the eight mutations, it was evident that the three mutations causing frameshifts and/or early termination signals (Table 1, mutation numbers 1, 2, and 5) could not produce active TPO. To verify whether the other five mutations indicated by an asterisk (Table 1, mutation numbers 3, 4, and 6–8) were responsible for the organification defects, we investigated the impact of these mutations on TPO expression and activity.

Materials and Methods

Mutagenesis

To obtain a full-length TPO complementary DNA (cDNA) probe, a 2.6-kilobase *EcoRI/BglII* fragment of TPO 3 cDNA (14) was cloned in pAlter-1 (Promega, Madison, WI). After digestion with *Sall*, a blunt site in the polylinker was created using the Klenow fragment of DNA Polymerase I (15) followed by digestion with *SfiI*. Thyroid cDNA generated by RT-PCR using oligo pd(N)₆ (10) was amplified using primer 1F1 (5'-ACTCAGCAGTGCAGTTGGCT-3') and 8R1 (5'-ACGCGGAG-CAGCCCTTCGGC-3') to generate the 5' part of the human TPO cDNA. This cDNA fragment was treated with the Klenow fragment of DNA polymerase I and digested with *SfiI*. The 875-bp fragment containing the 5' part of TPO cDNA was cloned into pAlter-1 containing the 3' part of TPO cDNA. Six clones were sequenced on the ABI Prism 377 DNA sequencer using the dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA) for PCR errors in the first 900 bps. Further sequencing revealed a polymorphism at position 2088 described by Bikker *et al.* (11) and Kimura *et al.* (13). Other single nucleotide substitutions (G → A) at position 1169 (Gly → Asp) (not present in 25 unrelated individuals) and a (G → A) at position 1374 introducing a

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TABLE 1. TPO mutations in 13 Dutch TIOD families

Exon	Mutation	Position	Distribution	Nature of mutation	No.
2	+ 20 bp	141	3	Frameshift ^a	1
8	+ GGCC	1277	7	Frameshift ^b	2
9	A → T	1429	1	Ile 447 Phe*	3
				ag/AT → ag/TT	
9	T → G	1447	6	Tyr 453 Asp*	4
10	C → T	1708	2	Arg 540 stop ^c	5
10	G → A	1858	4	Gly 590 Ser*	6
				AG/gt → AA/gt	
14	G → A	2485	1	Glu 799 Lys*	7
14	+ C	2505	1	Frameshift*	8

Distribution of inactivating mutations in 25 affected TPO alleles. All mutations are described by Bikker *et al.* (11) and ^a Bikker *et al.* (9), ^b Abramowicz *et al.* (12), and ^c Bikker *et al.* (10). Numbering is according to Kimura *et al.* (13).

*, Mutations indicated with an asterisk (3, 4, and 6–8) are expressed using the SFV expression system.

TABLE 2. Sequence of oligonucleotides used for site-directed mutagenesis

Primer	Sequence
mut8/A 1374 G	5' - CGGCGTCCGCGCTCCAGTGCAGCATTGA - 3'
mut9/A 1429 T	5' - TCAGGGTGATGAAGTGGTGCAGAGG - 3'
mut9/T 1447 G	5' - TCCCAGGATCCTGGGGATATCATCTCTCAGGGTGATGATCTGG - 3'
mut10/G 1858 A	5' - TCATTGTAACTTGGCAGCCCG - 3'
mut14/G 2485 A	5' - TCTGCACACTTGTTCACATCT - 3'
mut14/2505 + C	5' - GGCCTGGCAGGGGGGGTGGGCACCG - 3'

The numbers behind the slash are positions of mutated nucleotides (13). The first number indicates the involved exons. Mutated nucleotides are underlined.

termination signal were also detected. These mutations originated from the clone described by Libert *et al.* (14). The original sequence (13) at 1374 was restored using the Altered Sites II *in vitro* Mutagenesis System (Promega) using primer mut8/A 1374 G (Table 2). The restored clone pAlter/TPO 3.1 was used to generate the mutated TPO clones also by the Altered Sites II system. Primer sequences are shown in Table 2. The primer introducing the mutation at position 1447 was more complicated than the other ones. To prevent severe internal hybridization of this primer, two silent mutations were introduced creating an internal *EcoRV* restriction site, which is useful for convenient screening of mutants. The mutated cDNA clones were digested by *EcoRI*/*HindIII* isolated from low gelling agarose. Blunt ends were created by treatment with Klenow, and the fragments were subcloned into the alkaline phosphatase-treated *SmaI* site of pSemliki Forest Virus1 (SFV) (Gibco BRL, Life Technologies, Gaithersburg, MD). The orientation of the fragments was tested by *SfiI*/*SpeI* digestion, and the presence of the desired mutations was confirmed by sequencing. All sequences generated by PCR were checked by sequencing.

mRNA preparation and transfection

pSFV/TPO3.1 and the clones containing the mutated sequences were linearized by digestion with *SpeI*. This served as a template for recombinant RNA synthesis performed according to the conditions provided by the manufacturer (Gibco BRL, Life Technologies). RNA CAP Structure Analog was purchased from New England Biolabs (Beverly, MA), RNasin ribonuclease inhibitor from Promega, and SP6 RNA polymerase from Boehringer (Mannheim, Germany). Incubations were performed at 37°C for 1.5 h.

CHO-K1 cells (ATCC CCL 61) (American Type Culture Collection, Rockville, MD) were maintained in Ham's F12 medium supplemented with 10% FCS, penicillin (50,000 IU/L), and streptomycin (10 mg/L). RNA (20 µl) and 0.8×10^7 cells in cytomix (16) were electroporated (Easyject plus, Eurogentec, Seraing, Belgium) in a 2-mm cuvette. Electroporation was performed at 1500 microfarads and 260 V. Thereafter, the cells were plated in 10-cm dishes and for immunostaining in chamber slides (Nunc, Naperville, IL) and cultured for 24 h in the presence of 1 µg/mL hematin. For immunostaining, the cells were washed with PBS and fixed in 4% formalin in PBS for 10 min, followed by a 4-min incubation with chilled methanol (−20°C) and for 2 min in chilled acetone (−20°C). The cells were rehydrated in PBS and incubated for 15 min in

PBS containing 10% FCS. Slides were incubated with polyclonal anti-human TPO for 20 h (4°C). Excess antibody was removed by washing with PBS, followed by a 1-h incubation of fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin (4°C). After washing with PBS, slides were mounted with Vectashield (Vector Labs., Burlingame, CA).

Peroxidase activity measurement

Cells were harvested with a rubber scraper in 3 mL PBS, and protein concentration was determined on a 100-µl aliquot using the Bio-Rad protein assay (BioRad, München, Germany). The cells were then pelleted and subsequently suspended in 0.1% deoxycholate (0.2 mL/mg cellular protein) containing 1% aprotinin and incubated for 10 min at 4°C (4). The extract was microcentrifuged for 5 min, and the supernatant removed to measure enzymatic activity using the guaiacol and I_3^- assay (17). For the guaiacol assay, 50 µl of the supernatant was assayed in a final volume of 750 µl, containing 35 mM guaiacol and 0.5 mM H_2O_2 in 0.1 M Tris-HCl, pH 8.6. The absorbance at 470 nm was followed and activity was expressed as $\Delta A \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

For the I_3^- assay, 25 µl of the supernatant was used in a final volume of 750 µl containing 0.1 M potassium phosphate, pH 7.5, 50 mM potassium iodide, and 0.25 mM H_2O_2 . The absorbance at 353 nm was followed, and activity was expressed as $\Delta A \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Spectrophotometric analysis was done on a Shimadzu UV-200.

Western blotting

Thirty micrograms of the deoxycholate extracted membrane protein fraction containing recombinant TPO and normal control TPO (microsomal fraction) (10) were electrophoresed on a 7.5% SDS-polyacrylamide gel under reducing conditions. The gel was blotted onto nitrocellulose using a Bio-Rad Mini Protean 2 system (Bio-Rad Labs., Richmond, CA), followed by incubation with polyclonal rabbit antihuman TPO antibody (gift of Dr. J.P. Banga). This antibody was raised against TPO isolated from thyroid tissue of a Graves' disease patient. TPO protein was visualized using ECL Western blotting detection reagent (Amersham Life Science, Little Chalfont, U.K.).

Results

Expression of TPO

Human recombinant TPO was expressed in CHO-K1 cells using the expression system based on the SFV replication. Cells were harvested 24, 48, and 72 h after transformation. The level of TPO expression reached a maximum at 24 h (Fig. 1). Protein levels declined after 24 h, and at 72 h after transfection almost no TPO was observed (data not shown). Cells harvested 24 h after transfection displayed TPO protein expression independent of hematin addition. However, peroxidase activity was only observed when hematin was added to the medium.

TPO RNA of the mutants (Tables 1 and 2) was transfected and harvested as described in *Materials and Methods* and analyzed by Western blotting and immunofluorescence. All mutants expressed similar amounts of TPO antigens of about 110 kDa on the Western blot (Fig. 1). TPO expressed by mutant 2505+C (lane 7) was slightly shorter, caused by the frameshift introduced by the single nucleotide insertion at position 2505, which results in an altered stretch of 72 amino acids and an early termination signal in exon 16 (at amino acid 879). The predicted protein is about 6 kDa shorter.

The expression of wild-type and mutant recombinant TPO in the CHO-K1 cells was also visualized by immunofluorescence using a polyclonal antibody to human TPO (Fig. 2). Cells expressing wild-type TPO showed a clear fluorescein isothiocyanate signal concentrated on the cell surface (Fig. 2A), whereas no fluorescence was seen when pSFV1 RNA was transfected (Fig. 2C). The signal for all but one mutant was most intense on the cell surface, comparable with the image obtained in Fig. 2A. Only mutant 2505+C showed TPO antigens that were not restricted to the cell surface (Fig. 2B). This was not unexpected, because the membrane spanning part of exon 15, normally present as a hydrophobic peak on the Kyte-Doolittle plot was, as result of the frameshift, turned into a stretch of amino acids without elevated hydrophobicity.

Functional analysis

To measure wild-type and mutant enzyme activity, both the I_3^- - and guaiacol assays were carried out (Table 3). Wild-

FIG. 1. Western blot showing expression of wild-type and mutated recombinant TPO. Lane 1, TPO originating from normal thyroid tissue (microsomal fraction). Lane 2, Wild-type recombinant TPO. Lanes 3–7, Recombinant TPO containing mutation numbers 3, 4, 6, 7, and 8 (see Table 1). Lane 8, pSFV1 RNA transfected CHO-K1 cells (control).

FIG. 2. Immunostained CHO-K1 cells expressing wild-type recombinant TPO (A), TPO containing mutation 2505+C (B), and pSFV1 RNA (control) (C).

type recombinant TPO showed enzymatic activity in both assays. TPO generated by recombinant G 1858 A RNA transfected CHO cells, displayed comparable enzyme activity. In the other mutated TPO RNA transfected CHO cells, TPO activity was below detection level. Measurement of TPO activity using the I_3^- assay was only possible when 50 mM potassium iodide was present in the assay. These reaction conditions resulted in a relatively high nonenzymatic reaction rate, giving a high background.

TABLE 3. Guaiacol and iodide oxidation activity of expressed human TPO protein^a

Mutation	Guaiacol oxidation	Iodide oxidation
Wild type	0.77 ± 0.07	0.90 ± 0.05
A 1429 T	ND	*
T 1447 G	ND	*
G 1858 A	0.63 ± 0.07	0.89 ± 0.27
G 2485 A	ND	*
2505 + C	ND	*
Control	ND	*

^a Guaiacol and iodide oxidation activity was measured as described in *Materials and Methods*. Values represent specific activity expressed in $\Delta A \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Activity determinations were based on single determinations of two separate transfection rounds with separate electroporations in triplo. Values are means $\pm$ SD. Values for iodide oxidation activity are observed rates minus nonenzymatic rate.

*, Comparable with nonenzymatic reaction rate (0.51 ± 0.24). ND, No detectable activity (<10% of wild-type expressed TPO).

Discussion

Expression of recombinant TPO by the SFV system allowed us to verify whether the mutations occurring in patients with total iodide organification defects were in fact inactivating peroxidase activity or just linked polymorphisms. All mutants were expressed as immunoactive TPO, and four of the five mutants were enzymatically inactive in both the guaiacol and iodide assay (Fig. 1 and Table 3). This is a strong indication that the mutations are present at crucial positions of the TPO gene, resulting in inactivated TPO.

The cysteine and histidine residues in the vicinity of the heme group are located in highly conserved regions of TPO, lactoperoxidase (LPO), myeloperoxidase (MPO), and eosinophil peroxidase (EPO), although the overall amino acid content was identical for only about 45% (18, 19). The mutations in exons 9 and 14 (Table 1) were located in these conserved regions of the TPO gene. Aromatic substrate binding sites are supposed to be essential for peroxidase activity. In LPO a tyrosine binding site was demonstrated near the heme group (20). In MPO, a hydrophobic pocket at the entrance of the distal heme cavity (composed of Arg, His, and Gln) has been demonstrated. In this hydrophobic pocket aromatic substrates such as Tyr or Phe can bind. Three Phe residues are involved in the aromatic substrate binding site to the distal cave of MPO (21). These amino acids are all in identical positions in TPO, LPO, MPO, and EPO, confirming their essential function.

Kimura and Ikeda-Saito (22) suggested that in TPO the proximal His, which is linked to the ironcenter of the heme, is located at residue 407. However, x-ray crystallography at 3 Å of MPO clearly showed that His-336 is more likely to act as proximal His, because it was located within 4.5 Å of the heme (19). The corresponding His in TPO is located at position 494 in exon 9. The mutations A 1429 T and T 1447 G in exon 9, resulting in the gain (Ile 447 Phe) or loss (Tyr 453 Asp) of an aromatic ring in the neighborhood of the heme binding site, may therefore strongly interfere with heme binding, altering the hydrophobic pocket and/or influencing the electron transfer in the environment of the catalytic center.

Less is known of the structure at the carboxyl terminus of

TPO. MPO, EPO, and LPO all lack the corresponding part of the TPO protein (encoded by exons 13–17) containing the membrane-spanning part (encoded by exon 15). Inactivating mutations G 2486 A and 2505+C (Table 1) were both located in exon 14. Recombinant TPO containing the 2505+C mutation, leading to TPO in which the membrane-spanning domain was replaced by a novel stretch of amino acids, was at least partly retained in the CHO cells as shown by immunostaining of the transfected cells, suggesting that routing or protein folding is affected (Fig. 2B). Although a secreted form of TPO (missing the membrane-spanning part) described by Foti *et al.* (6) was enzymatically active, our mutant was inactive and retained in the cells, however, some excretion could not be excluded. Because TPO truncated by an insertion at –126 (2505+C) is inactive, and TPO truncated at –85 as described by Foti *et al.* (6), was enzymatically active, the region between –85 and –126 amino acids may be important for enzyme activity.

The result of the Glu 799 Lys substitution (mutation G 2486 A), an acidic amino acid (Glu) alteration to a basic (Lys) amino acid, is also enzymatically inactive TPO. The cellular distribution of this mutant TPO was comparable with wild-type recombinant TPO (Fig. 2A), indicating that membrane binding was not affected.

Both mutations in exon 14 (G 2486 A and 2505+C) yielded enzymatically inactive TPO, suggesting that this region of the protein is important for proper folding and does not only serve as a hinge for insertion of the membrane.

Recombinant Gly 590 Ser TPO (G 1858 A) (Table 1, mutation number 6) expressed enzymatic activity in the guaiacol and I_3^- assay. This mutation was cosegregating with TIOD in three unrelated families, homozygous in one family, and heterozygous in two other unrelated TIOD families, in combination with exon 9 mutations A 1429 T or T 1447 G. The possibility that mutation G 1858 A, which is present at the exon 10/intron 10 border, inactivates the TPO genes of the patients by alternative splicing is a good possibility. At least three other G $\rightarrow$ A mutations at the 5' end of an exon led to exon skipping, and the exon immediately preceding the mutation was removed from the RNA transcript (23). In the case of the mutation G 1858 A in TPO, exon 10 could be skipped, yielding inactive TPO-2 (10). Unfortunately thyroid tissue was not available to test this hypothesis. We cannot exclude the possibility that the G 1858 A mutation is a linked polymorphism in a defective TPO gene carrying a mutation we missed in the denaturing gradient gel electrophoresis screen, however, we consider this highly unlikely.

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