Cloning of Two Human Thyroid cDNAs Encoding New Members of the NADPH Oxidase Family*

Received for publication, February 4, 2000, and in revised form, March 21, 2000
Published, JBC Papers in Press, May 9, 2000, DOI 10.1074/jbc.M000916200

Xavier De Deken‡‡, Dantong Wang‡, Marie-Christine Many†, Sabine Costagliola‡, Frédérick Libert‡‡, Gilbert Vassart‡‡*, Jacques E. Dumont‡‡, and Françoise Miot‡‡‡

From the †Institut de Recherche Interdisciplinaire and **Service de Génétique Médicale, Hôpital Erasme, Université Libre De Bruxelles, Campus Erasme, 808, route de Lennik, 1070 Bruxelles and ‡Laboratoire d’Histologie, Université Catholique de Louvain, 1200 Bruxelles, Belgium

Two cDNAs encoding NADPH oxidases and constituting the thyroid H2O2 generating system have been cloned. The strategy of cloning was based on the functional similarities between H2O2 generation in leukocytes and the thyroid, according to the hypothesis that one of the components of the thyroid system would belong to the gp91Phox/Mox1 gene family and display sequence similarities with gp91Phox. Screening at low stringency with a gp91Phox probe of cDNA libraries from thyroid cells in primary culture yielded two distinct human cDNA clones harboring open reading frames of 1551 (ThOX1) and 1548 amino acids (ThOX2), respectively. The encoded polypeptides display 83% sequence similarity and are clearly related to gp91Phox (53 and 47% similarity). The theoretical molecular mass of 177 kDa is close to the apparent molecular mass of 180 kDa of the native corresponding porcine flavoprotein and the protein(s) detected by Western blot in dog and human thyroid. ThOX1 and ThOX2 display sequence similarities of 53% and 61%, respectively, with a predicted protein of Caenorhabditis elegans over their entire length. They show along their first 500 amino acids a similarity of 43% with thryperoxidase. The corresponding genes of ThOX1 and ThOX2 are closely linked on chromosome 15q15.3. The dog mRNA expression is thyroid-specific and up-regulated by agents activating the cAMP pathway as is the synthesis of the polypeptides they are coding for. In human thyroid the positive regulation by cAMP is less pronounced. The proteins ThOX1 and ThOX2 accumulate at the apical membrane of thyrocytes and are co-localized with thryperoxidase.

Iodine enters the thyroid as iodide through an active transport process mediated by the Na+−/symporter NIS (1−3). Iodide is oxidized to a higher oxidation state before it acts as an effective agent capable of iodinating thyroglobulin, the precursor of thyroid hormones (4). Both the iodination of thyroglobulin and the oxidative coupling of the resulting iodotyrosines into iodothyronines take place in the follicular space in close contact with the apical pole of thyrocytes. According to current knowledge three different systems are co-localized within the apical plasma membrane and cooperate to the hormonogenic reaction: an iodide transporter, recently identified as the product of the pendrin gene (5, 6), thryperoxidase (7, 8), and an H2O2 generating system. H2O2 is the limiting factor in the oxidation of iodide in the synthesis of thyroid hormones reaction (9).

H2O2 is formed from the oxidation of NADPH by an NADPH oxidase, a flavoprotein that has been partially purified from solubilized pig thyroid plasma membrane (10). It is known that the enzyme requires Ca2+ to be fully active, but the precise molecular structure of the H2O2 generating system operating in the thyroid remains ill defined. In polymorphonuclear neutrophils, an NADPH oxidase activity, with functional similarities to the thyroid system, is responsible for the burst of oxygen consumption (“respiratory burst”). It catalyzes the production of superoxide anions (O2−) in activated leukocytes. In these cells the molecular species implicated have been well defined; they comprise a glycoprotein, gp91Phox, and a smaller 22-kDa protein, p22Phox located at the membrane and at least three cytosolic components (p40Phox, p41Phox, and p67Phox) migrating to the membrane after stimulation of the cell (11). In nonphagocytic mammal cells, Mox1 has been identified as another superoxide generating NADPH oxidase belonging to the same family as gp91Phox. It has been implicated in mitogenesis and cancer (12). More recently NOH-1, generated from Mox1 transcripts by alternative splicing, has been described as a mammalian H+ channel (13).

From the functional similarities between H2O2 generation in leukocytes and the thyroid, we based a cloning strategy on the hypothesis that one of the components of the thyroid system would belong to the gp91Phox/Mox1 gene family and, accordingly, display sequence similarities with gp91Phox. Screening of cDNA libraries from thyroid cells in primary culture, at low stringency with a gp91Phox probe, yielded two distinct cDNA clones harboring open reading frames of 1551 (ThOX1) and 1548 amino acids (ThOX2), respectively. The encoded polypeptides display 83% sequence similarity and are clearly related to gp91Phox (53 and 47% similarity). The two corresponding genes are closely linked on chromosome 15q15.3. Their transcripts are thyroid-specific, subjected to positive regulation by cAMP agonists, and encode polypeptides accumulating at the apical membrane of thyrocytes.

During the completion of the present study, Dupuy et al. (14), using a strategy based on the purification of the enzymatic activity, reported cloning of a single cDNA encoding a 1210-residue thyroid NADPH oxidase. Comparison with the present...
results demonstrate that their p138\textsuperscript{T\textsubscript{ox}} corresponds to a carboxyl fragment of ThOX2, lacking the first 338 residues.

**EXPERIMENTAL PROCEDURES**

** Screening of Thyroid cDNA Libraries—**A ZapII cDNA library was constructed as described (15) with polya\(^{+}\) RNA prepared from human thyroid cells maintained in primary culture for 5 days with TSH.\(^1\) 18 x 10\(^6\) recombinant phages were screened at low stringency (hybridization at 42 \(^\circ\)C; 30% formamide, 6 x SSC (1 x SSC = 0.15 m NaCl, 0.015 m sodium citrate), 5 x MTA, pH 8, 0.5% SDS, 0.25% milk powder; washings at 50 \(^\circ\)C; 2 x SSC, 0.1% SDS) with a 1.3-kilobase PstI-EcoRI fragment of gp91\textsuperscript{Phox} cDNA (a kind gift of Prof. S. Orkin). Two different 1,1-kilobase clones were obtained. Segments corresponding to their 5’ portions of complete (first injection) or incomplete (subsequent injections) gpl11 cDNA libraries made from human (16) and dog (17) thyroids, respectively. Segments containing the human counterparts of the canine coding sequences were obtained by polymerase chain reaction (ThOX1: forward 5’-GACGGAATTCTATCCATCGGCGCTTC-3’, reverse 5’-ACTACTCCAGCTGGAGAACTTGAGTTCCGA-3’; ThOX2: forward 5’-GTCGACGCGATTAGGTTGCG-3’, reverse 5’-ACTACTCGAGACCTCAATTCTGCTC-3’). The 5’ noncoding sequence of each human cDNA was obtained by a 5’ rapid amplification of cDNA ends, starting from 1 μg of polya\(^{+}\) RNA of human thyrocytes in culture and three specific primers (ThOX1 5’-GGATGCGGATGTTGAGGAACT-3’, CCA-CGTCACAGGTTGCGAAA, 5’-CCCAACACTTGCGCTTTCTC-3’, ThOX2: 5’-GTCGACGCGATTAGGTTGCG-3’, reverse 5’-ACTACTCGAGACCTCAATTCTGCTC-3’; EcoRI fragment of ThOX1 and a 1427-bp KpnI-EcoRI fragment of ThOX2. The human probes were a 687-bp Sau3A I-EcoRI fragment of ThOX1 and a 1628-bp HindII-Xhol fragment of ThOX2. Filters were washed four times for 30 min in 0.1 x SSC, 0.1% SDS at 65 \(^\circ\)C.

**Immunodetection—**A rabbit polyclonal antibody was raised against the Arg618–His1044 fragment of ThOX1 produced in E. coli by the pET-24d vector system (Novagen). New Zealand White rabbits (Iffa Credo) were subcutaneously injected three times, at a 3-week interval, with 150 μg of recombinant ThOX1 fragment, in the presence of complete (first injection) or incomplete (subsequent injections) Freund’s adjuvant (Sigma). Sera were collected 10 days after the third injection and were directly used at a dilution of 1:10,000 in Western blot analyses and 1:200 in immunohistochemistry experiments.

**Western Blot—**Protein extracts were obtained by lysis in classical Laemmli buffer of thyrocytes or Cos-7 cells transfected with ThOX1 cDNA in pcDNA3 and Fugene (Roche Molecular Biochemicals). Proteins were separated by SDS/polyacrylamide gel electrophoresis on 6 or 7.5% polyacrylamide gels. 10–30 μg of protein extracts, measured by a protein assay (BioRad), were loaded, and the resolved proteins were transferred to nitrocellulose. Immune complexes were detected with a horseradish peroxidase-coupled anti-rabbit IgG antibody (Amersham Pharmacia Biotech) according to the ECL method (NEN Life Science Products).

**Protein Localization by Immunohistochemistry—**Human paranoval tissue was obtained at surgery and quickly frozen in liquid nitrogen. After inhibition of endogenous peroxidase activity by addition of 0.228% periodic acid for 45 s, the frozen sections were incubated for 1 h with the anti-ThOX1 antibody diluted at 1:200 and for 1 h with a second anti-rabbit immunoperoxidase-conjugated antibody. Antibody binding was revealed using diaminobenzidine tetrachloride. Controls were performed by using preimmune serum instead of the first antibody, by omission of the first antibody, or by omission of the first and second antibodies.

\(^1\)The abbreviations used are: TSH, thyrotropin; EGF, epidermal growth factor; bp, base pair(s).

Thyroperoxidase was immunolocalized using the N-vision technique. The first mouse monoclonal antibody (gift from Dr De Mico) was added on the frozen sections for 1 h at dilution 1:500.

The secondary antibody is a goat anti-mouse immunoglobulin conjugated to peroxidase-labeled polymer (Dako En Vision +). The chromogen used for revelation was AEC giving a red staining (3-amin-9 ethylcarbazole).

**Thyroid Cell Culture—**Follicles were isolated by collagenase digestion and differential centrifugation from fresh dog and human thyroid as described previously (21, 22). The follicles were seeded in 60- or 90-mm tissue culture-treated Petri dishes and cultured for 4 days in control medium. This procedure yields cultures containing thyrocytes pure at 99% (23). The cells were stimulated for different times with 10 μM forskolin (Calbiochem), 1 milliunit/ml TSH (Sigma), or 25 ng/ml EGF (Sigma).

**RESULTS**

**Molecular Cloning of Two cDNA Encoding Proteins of the Flavoprotein Family—**Screening by hybridization at low stringency of two human thyrocyte cDNA libraries with a gp91\textsuperscript{Phox} cDNA segment yielded systematically two types of clones displaying significant similarity with the probe. When sequenced and aligned, they contained an uninterrupted reading frame corresponding to the 3’ region of gp91\textsuperscript{Phox}, and none of them presented an initiation codon in a favorable context. After several unsuccessful attempts to isolate full-length cDNAs from human libraries, a dog \(\lambda\) gt11 cDNA library known to contain large inserts was screened with the most 5’ portions of each type of clones. This yielded canine homologs of the two categories of human clones which, when aligned with gp91\textsuperscript{Phox}, displayed open reading frames extending upstream of gp91\textsuperscript{Phox} initiation codon. Reverse transcription-polymerase chain reaction with a human-specific reverse primer and a canine forward primer (see “Experimental Procedures”) allowed extension of the 5’ portions of the two human cDNA clones to a total length of 5,369 and 6,126 bp, respectively. Finally 5’-rapid amplification of cDNA ends polymerase chain reaction with primers specific of each sequence yielded two presumably complete cDNA sequences: the first was 5,693 bp long (GenBank\textsuperscript{TM} accession number AF230498) with the potential to encode a protein of 1,551 amino acids (ThOX1 for thyroid oxidase 1); the second was 6,410 bp long (GenBank\textsuperscript{TM} accession number AF230496) and contained an open reading frame of 1,548 amino acids (ThOX2).

The primary structures of ThOX1 and ThOX2 showed 83% similarity (Fig. 1A). The molecular weight of the predicted unglycosylated proteins was 177,000. According to von Heijne’s algorithm (24), both contained a putative signal peptide with Asn\(^\text{23}\) and Gin\(^\text{26}\) being the preferred candidates for the N terminus of the mature proteins in ThOX1 and in ThOX2, respectively. Both proteins contained two putative EF-hand motifs, \(\text{GKDNGYLSFRE}^{\text{460}}\) and \(\text{GKDNGGKISFRP}^{\text{1172}}\) in ThOX1 and \(\text{GKDNGYLSFRE}^{\text{464}}\) and \(\text{GKDNGGKISFRP}^{\text{1168}}\) in ThOX2. The sequence of the 1210 last amino acids of ThOX2 was identical to that of p138\textsuperscript{T\textsubscript{ox}}, a recently reported cDNA claimed to encode a component of thyroid \(\text{H}_{2}\text{O}_{2}\) generating system (14). When compared with p91\textsuperscript{Phox} and Mox1, ThOX1 and ThOX2 displayed 53 and 47% similarity with the former and 54 and 47% identity with the latter, respectively. The similarity was, however, limited to their last 569 C-terminal residues, because both of them contained an N-terminal extension with no counterpart in p91\textsuperscript{Phox} (or Mox1) (982 and 979 residues in ThOX1 and ThOX2, respectively). The homology with p91\textsuperscript{Phox} allowed, in both ThOX1 and ThOX2, identification of a conserved putative NADPH-binding site (1389GIVTPP1395, 1420WVTR1424, 1486GLRSLITHFGFR1495 and 1517VFSCGFP1522) in ThOX1 and 1386GIVTPP1393, 1417WVT-R1421, 1480GLRSLITHFGFR1492, and 1514VFSCGFP1519 in ThOX2), a putative FAD-binding site 1319HPTTLS1325 in ThOX1 and
Cloning of Two Thyroid cDNAs Encoding NADPH Oxidases

A

Fig. 1. Primary structure of ThOX1 and ThOX2 and sequence comparison. A, deduced amino acid of ThOX1 (GenBank™ accession number AF230495) and ThOX2 (GenBank™ accession number AF230496) aligned with C. elegans predicted protein (GenBank™ accession number AF043697). Shaded boxes indicate identical residues, and + denotes the position of the four histidines and arginine conserved in gp91Phox. Putative FAD-binding and NADPH-binding domains and EF-hand regions are indicated with dotted lines. The seven putative transmembrane regions are shown with asterisks. B, presumed structure of ThOX protein; model of the transmembrane topology and putative functional domains.
Cloning of Two Thyroid cDNAs Encoding NADPH Oxidases

Asn382, Asn455, and Asn537 in ThOX2). The N-terminal region harbors five putative sites of N-glycosylation (Asn94, Asn342, Asn354, Asn461, and Asn534 in ThOX1 and Asn106, Asn348, Asn382, Asn455, and Asn537 in ThOX2).

The sequence organization of ThOX1 and ThOX2 is shared by a predicted protein of Caenorhabditis elegans (GenBank\textsuperscript{TM} accession number AF043697) found by sequencing of chromosome III of C. elegans (26), with which they display respectively 53 and 61\% similarity over their entire length. Surprisingly, stretches of amino acids displaying significant sequence similarity with proteins belonging to the peroxidase gene family were found both in the C. elegans protein and in ThOX1,2 in the N-terminal extensions (43\% similarity with thyroperoxidase over 500 residues). The hydropathy profiles (TMpred program (27)) of ThOX1 and ThOX2 include seven hydrophobic stretches that could be membrane spanning regions. Considering that Ca\textsuperscript{2+} binding to EF-hands motifs as well as NADPH and FAD binding presumably occur in the intracellular portions of the protein, we propose a common structure for ThOX1 and ThOX2 shown in Fig. 1B.

The genes corresponding to ThOX1 and ThOX2 have been co-localized on chromosome 15q15.3 using the radiation hybrid panel method of Genebridge 4 (Research Genetics). Comparison of their nucleotide sequence with the current human genomic data base (high throughput genomic sequences, NCBI, National Institutes of Health) revealed stretches of identity corresponding to exons in the partially sequenced 163_P_10 clone on chromosome 15 (AC 009700.3, release of October, 1999). This allowed positioning of at least 20 intron-exon junctions in each gene and confirmed their location in close proximity on chromosome 15.

Distribution and Regulation of mRNA Accumulation—The presence of ThOX1 and ThOX2 mRNA has been explored in 12 different dog tissues by Northern blotting (liver, lymph node, heart, brain, testis, kidney, cerebellum, pancreas, stomach, spleen, and thyroid). Except for a very weak ThOX2 signal in the stomach, it was exclusively found in the thyroid (Fig. 2).

Regulation of ThOX1 and ThOX2 gene expression was studied in dog and human thyroid cells in primary culture after stimulation for up to 72 h with TSH (1 milliunit/ml), forskolin (10\textsuperscript{-5} M), or EGF (25 ng/ml). In dog and human thyrocytes, Northern blotting revealed transcripts estimated at 5.7 and 6.4 kilobases when hybridizations were performed with the 3\textsuperscript{\prime} region of ThOX1- and ThOX2-specific probes, respectively. 10\textsuperscript{-5} M forskolin stimulated accumulation of both ThOX1 and ThOX2 mRNAs of dog thyrocytes after 48 h with a further increase after 72 h (Fig. 3B). This stimulation was reproduced with TSH (Fig. 3C). EGF had no effect on the mRNA expression. In human thyrocytes the up-regulation by forskolin was less pronounced (Fig. 3A). The same signal was observed when the blots were hybridized with the 5\textsuperscript{\prime}-extremity of the ThOX1 (700 bp) and ThOX2 (1200 bp) cDNAs (not shown).

Localization and Regulation of ThOX1 Protein—The polyclonal antibody raised against a fragment of ThOX1 made it possible to localize ThOX1 in the supranuclear apical pole of all thyroid cells. In some cells the labeling was more pronounced on the apical plasma membrane (Fig. 4A). No labeling was obtained when using preimmune serum instead of anti-ThOX1 (Fig. 4B). Localization of ThOX1 protein is similar to that of thyroperoxidase also detected in the apical pole of the thyroid cells (Fig. 4C).

Western blot analysis of lysates of Cos-7 cell transfected with an expression plasmid coding for human ThOX1 detected a major immunoreactive band corresponding to a protein with an apparent molecular mass of 180 kDa (Fig. 5). This mass was close to the theoretical value calculated from the predicted amino acid sequence, the difference being most probably accounted for by \(N\)-linked carbohydrate chains. The antibody recognized a protein of the same molecular mass in three rat

**Fig. 2.** ThOX1 and ThOX2 mRNA expression in dog tissues. Total RNA was extracted from different dog tissues. Lane 1, liver; lane 2, lymph node; lane 3, heart; lane 4, lung; lane 5, brain; lane 6, testis; lane 7, kidney; lane 8, cerebellum; lane 9, pancreas; lane 10, stomach; lane 11, spleen; lanes 12–14, thyroid. 10 \(\mu\)g of total RNA were subjected to Northern blot analysis and hybridized with a cDNA probe of ThOX1 or ThOX2. The bottom panel shows acridine orange staining of ribosomal RNA (rRNA).

**Fig. 3.** ThOX1 and ThOX2 mRNA expression after chronic stimulation with forskolin (10\textsuperscript{-5} M), TSH (1 milliunit/ml), or EGF (25 ng/ml). Total RNA was extracted, and 10 \(\mu\)g were subjected to Northern blot analysis. Panel a shows the mRNA expression in the human thyrocytes cultured for 24 or 48 h with forskolin. mRNA expression is measured in dog thyrocytes cultured for 8, 24, 48, or 72 h with forskolin or EGF (panel b) and for 72 h with TSH (panel c). The bottom panels of the figure show acridine orange staining ascertaining that equal amount of RNA was spotted in each lane (45). C, control; Fsk, forskolin; kb, kilobases.
thyroid cell lines: FRTL-5, WRT, and PCCL3. The protein was also detected in dog and human thyrocytes. Western blots made with thyroid tissues revealed two bands corresponding to proteins with molecular masses of 180 and 190 kDa (Fig. 5). Prior incubation of the antibody with 10 μg of the recombinant ThOX1 fragment used to immunize the rabbit suppressed completely and specifically the bands. Stimulation of dog thyrocytes in culture by forskolin for 48 or 72 h resulted in a steady increase of the accumulation of the immunoreactive material. EGF had no effect even at 72 h. In human thyrocytes a weak stimulation by forskolin was observed at 72 h.

**DISCUSSION**

Using a strategy based on the functional homology existing between the NADPH oxidase systems in the thyroid and the polymorphonuclear neutrophil, we cloned from cDNA libraries of thyroid cells in primary culture, two different cDNAs displaying convincing sequence similarity with gp91Phox over their entire C-terminal portion (Fig. 1A). It should be noted that previous attempts using cDNA libraries made from whole thyroid tissue has only yielded the cDNA of gp91Phox. The available arguments to identify the two candidates as components of the thyroid H2O2 generating system include: 1) the level of sequence similarity with gp91Phox (53 and 54% for ThOX1 and ThOX2, respectively) which, apart from indicating a common ancestor (see below), is strongly suggestive of a similar function; 2) the almost exclusive thyroid specificity of the two transcripts (Fig. 2) and the co-localization of ThOX1,2 polypeptides with TPO at the apical pole of thyrocytes (Fig. 4); and 3) the perfect sequence identity of the last 1210 residues of ThOX2 with p138Tox, a thyroid NADPH oxidase reported recently by Dupuy et al. (14) as the catalytic moiety of the thyroid H2O2 generating system. Because this clone was obtained from the sequence of endoproteinase Lys-C peptides generated from a 180-kDa protein displaying NADPH oxidase activity (10), the most likely explanation is that the p138Tox clone of Dupuy et al. (14) is an incomplete version of ThOX2 lacking 1014 bases of coding sequence at its 5’ end. In contrast, ThOX2 would encode a full-size 180-kDa NADPH oxidase polypeptide (see Western blot on Fig. 5). The 83% sequence similarity between ThOX2 and ThOX1 strongly suggests that the latter would also encode a thyroid NADPH oxidase. The presence in the protein sequence of conserved FAD-, NADPH-, and heme-binding sites of the gp91Phox fits with the known biochemistry and physiology of the thyroid (28). Finally the regulation of mRNA expression of ThOX1,2 in dog thyroid cells in culture is in total agreement with the previous findings on the functional generation of H2O2 by these cells (29). Despite these convincing arguments, the definite proof that ThOX1 and ThOX2 are components of the thyroid H2O2 generating system will await reconstitution of these cells. This may require co-transfection of constructs encoding additional components that remain to be identified (10).

In their present state, sequence data bases contain five sequences displaying similarity with NADPH oxidases: gp91Phox, ThOX1, ThOX2, Mox1, and a conceptual protein of C. elegans.
Together, they constitute a subfamily of genes encoding flavoproteins. These flavoproteins contain NADPH- and FAD-binding domains, four specific histidines, and a conserved arginine involved in the binding of the heme prosthetic group. gp91^Phox possesses a NADPH oxidase activity, which catalyzes the production of superoxide anions (O$_2^-$) by the one-electron reduction of oxygen using NADPH as the electron donor. The cloning of members of the flavoprotein family, restricted to gp91^Phox two years ago, has been extended today to new members that are expressed in nonphagocytic cells; Mox1 presumably involved in cell proliferation and expressed in colon and vascular smooth muscle cells (12) and NOH, a mammalian H$^+$ channel that is in fact a spliced form of Mox1 (13).

Alignment of the various members of the protein family allows to classify them in two categories on the basis of the presence of two different building blocks. All five contain amino acid residue homologous segments, the NADPH- and FAD-binding sites, with the potential to encode an NADPH oxidase. Only the C. elegans protein and both ThOX1 and ThOX2 contain, in addition, a 500-residue N-terminal extension with sequence similarity with peroxidases. The structure of the NADPH oxidase segment has been modelled in gp91^Phox (25), which allows proposal of a possible structure for the corresponding portion of the ThOX proteins. The presumed structure of this portion of the proteins (Fig. 1B) shows a large first intracellular loop containing two EF-hand domains probably involved in the direct activation of the enzyme by Ca$^{2+}$ as demonstrated in porcine and human thyroid membrane fractions (30, 31), in intact follicles (32), and in thyroid slices (9, 33). The four histidines and the arginine involved in the heme binding in the gp91^Phox (25) are conserved in ThOX1 and ThOX2 as the FAD- and NADPH-binding sites. These sites are the presumed functional domains of the thyroid NADPH oxidase and should be located at the intracellular side of the apical pole of the thyrocyte.

The N-terminal portions of the ThOX polypeptides and the C. elegans predicted protein display homology with peroxidases and harbor three conserved potential sites for N-glycosylation. They are expected to be extracellular. Indeed, the EF-hand containing segment of the NADPH oxidase domain, in ThOX1 and ThOX2, is separated from the peroxidase homology domain by a hydrophobic segment presenting the characteristics of a transmembrane helix. This structural difference between the ThOX proteins and gp91^Phox could perhaps be related for an expected functional difference; the transfer of electrons from NADPH to oxygen results in the neutrophil in the formation of superoxide anions, whereas in the thyroid, H$_2$O$_2$ is formed directly (34).

In evolutionary terms, the presence of both the NADPH oxidase and the peroxidase homology domains in the ThOX and the C. elegans proteins indicates that a gene fusion event, at the origin of these proteins, predates the separation between vertebrates and invertebrates. The absence of the peroxidase domain in gp91^Phox is compatible with two scenarios; either this domain has been lost after the gene duplication event having generated the ThOX genes in the vertebrate lineage or the gp91^Phox gene evolved from an ancestral gene devoid of the peroxidase domain, which would have been lost in C. elegans. The relatively high sequence similarity between the ThOX genes in their NADPH oxidase domains and gp91^Phox strongly favors the first scenario. The co-localization of the two ThOX genes on chromosome 15q15.3, as determined by the radiation hybrid method, was confirmed by current information in the genomic data base; the two genes are very closely linked, perhaps in tandem, within a 153,400-bp bacterial artificial chromosome in the process of being fully sequenced.

Evolution of genes by fusion of segments encoding functional entities, which may remain separate in other genes, has been described (35, 36). The organization of iodide and thyroid hormonogenesis make use both of ThOX proteins, containing an NADPH oxidase and a peroxidase homology domain, and thyroperoxidase. Considering the toxicity of H$_2$O$_2$, association of two enzymes in a complex allowing channeling of H$_2$O$_2$ would make physiological sense (37–39). Future dissection of structure-function relationships of the ThOX proteins will be needed to identify the respective roles and possible interactions of thyroperoxidase and the N-terminal peroxidase homology domain of ThOX1 and ThOX2.

The existence of the two distinct ThOX genes raises the question of their respective roles. Both are expressed in a thyroid-specific manner and controlled in a similar way by TSH. Among other possibilities, they could constitute redundant entities or be implicated in a heteropolymeric structure, with each subunit playing a distinct role in the catalytic reaction. In the first case, with four copies of ThOX per diplod genome, the system should be particularly resistant to alteration by loss-of-function mutations. On the contrary, if a hetopolymeric structure is involved, depending on the stoichiometry of the complex, one could observe dominant negative effects in heterozygotes with mutations affecting the structure of the holoprotein. The rarity of cases with congenital defects of organization without mutations of the thyroperoxidase gene (40, 41) would rather argue in favor of the first hypothesis, but here also, clarification will require activity reconstitution.

Regulation of H$_2$O$_2$ generation is known to occur both at the gene expression and posttranscriptional levels. At the former level the results of Northern and Western blotting experiments with dog thyrocytes agree with biochemical data showing that the capacity of H$_2$O$_2$ formation is stimulated after a chronic elevation of cAMP (29). Although Northern blotting experiments clearly indicate that both ThOX1 and ThOX2 mRNAs are up-regulated by cAMP agonists, in the absence of antibodies specific for the individual proteins, it can only be concluded from Western blots that one or both proteins accumulate under the same conditions. In human but not in dog thyroid, TSH activates the phosphatidylinositol triphosphate-phospholipase C cascade. Acute stimulation of iodide organization and H$_2$O$_2$ production by the PIP2-phospholipase C cascade and by calcium has been observed in humans, dogs, and other species (9, 33, 42). The presence of the two EF-hands in the ThOX proteins provides a structural basis for these effects.

The successful cloning of human H$_2$O$_2$ generating system opens new perspectives in the physiopathology and diagnosis of thyroid diseases. It should allow to demonstrate the genetic lesion of this system in iodination defects observed in congenital hypothyroidism, thyroid cold adenomas, and thyroid cancer with normal thyroperoxidase (43, 44). The close proximity of thyroperoxidase and ThOX at the apical pole of thyrocytes suggests that autoimmune reactions leading to the generation of autoantibodies directed against the former might spread to the latter in Hashimoto’s thyroiditis or Graves’ diseases. By analogy with anti-thyroperoxidase autoantibodies, it is conceivable that some antibodies will have an inhibitory effect on the catalytic activity.

Acknowledgments—We thank the technician team of the Service de Génétique moléculaire for expert manipulations of the sequencers, Christiane Christophe for providing us the oligonucleotide primers, and Marie-Jeanne Simons for excellent technical advice.

REFERENCES
1. Dai, G., Levy, O., and Carrasco, N. (1996) Nature 379, 458–460
2. Eskandari, S., Luo, D. D. F., Dai, G., Levy, O., Wright, E. M., and Carrasco, N. (1997) J. Biol. Chem. 272, 27230–27238
3. Uyttersprot, N., Pelgrims, N., Carrasco, N., Gervy, C., Maenhaut, C., Dumont,
Cloning of Two Thyroid cDNAs Encoding NADPH Oxidases

J. E., and Miot, F. (1997) Mol. Cell. Endocrinol. 131, 195–203
4. Nunez, J., and Pommier, J. (1982) Vitamin Horm. 39, 175–229
5. Sheffield, V. C., Kruien, Z., Beck, J. C., Nishimura, D., Stone, E. M., Salameh, M., Sadeh, O., and Glaser, B. (1986) Nat. Genet. 12, 424–426
6. Scott, D. A., Wang, R., Kreman, T. M., Sheffield, V. C., and Karmishki, L. P. (1999) Nat. Genet. 21, 440–443
7. Taurog, A. (1996) in Werner and Ingbar's The Thyroid (Braverman, L. E., and Utiger, R. D. eds) pp. 47–84, Lippincott, Co., Philadelphia, PA
8. Alexander, N. M. (1977) Endocrinology 100, 1610–1620
9. Corvilaïn, B., Van Sande, J., Laurent, E., and Dumont, J. E. (1991) Endocrinology 128, 779–785
10. Gorin, Y., Ohayon, R., Carvalho, D. P., Deme, D., Leseney, A., Haye, B., Kaniaewski, J., Pommier, J., Virion, A., and Dupuy, C. (1996) Eur. J. Biochem. 240, 807–814
11. Bahier, B. M. (1999) Blood 93, 1464–1476
12. Suh, Y., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., Chung, A. B., Griendling, K. R., and Lambeth, J. D. (1999) Nature 401, 79–82
13. Banfi, B., Maturana, A., Jaconi, S., Arnaudeau, S., Laforge, T., Sinha, B., Ligeti, E., Demaurex, N., and Krause, K. (2000) Science 287, 138–142
14. Dupuy, C., Ohayon, R., Valant, A., Noel-Hudson, M., Deme, D., and Virion, A. (1999) J. Biol. Chem. 274, 37265–37269
15. Wilkin, F., Savonnet, V., Radulescu, A., Pommier, J., Virion, A., and Dupuy, C. (1996) Eur. J. Biochem. 240, 807–814
16. Nunez, J., and Pommier, J. (1982) Vitamin Horm. 39, 175–229
17. Sheffield, V. C., Kraiem, Z., Beck, J. C., Nishimura, D., Stone, E. M., Salameh, M., Sadeh, O., and Glaser, B. (1986) Nat. Genet. 12, 424–426
18. Scott, D. A., Wang, R., Kreman, T. M., Sheffield, V. C., and Karmishki, L. P. (1999) Nat. Genet. 21, 440–443
19. Taurog, A. (1996) in Werner and Ingbar's The Thyroid (Braverman, L. E., and Utiger, R. D. eds) pp. 47–84, Lippincott, Co., Philadelphia, PA
20. Minamide, L. S., and Bamburg, J. R. (1990) EMBO J. 9, 3111–3116
21. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
23. Marcotte, E. M., Pellegrini, M., Thompson, M. J., Yeates, T. O., and Eisenberg, D. (1999) Nature 402, 83–86
24. Enright, A. J., Iliopoulos, I., Kyripides, N. C., and Ouzounis, C. A. (1999) Nature 402, 86–90
25. Van Sande, J., Ketelhant-Balasse, P., Schell-Frederick, E., and Dumont, J. E. (1982) Mol. Cell. Endocrinol. 26, 31–40
26. Björkman, U., and Ekholm, R. (1995) Mol. Cell. Endocrinol. 111, 99–107
27. Hofmann, K., and Stoffel, W. (1993) EMBO J. 12, 4193–4196
28. Raspe, E., and Dumont, J. E. (1995) Endocrinology 136, 965–973
29. Deme, D., Virion, A., Ait Hammou, N., and Pommier, J. (1985) J. Biol. Chem. 260, 1158–1165
30. Leseney, A., Deme, D., Legué, O., Ohayon, R., Chanson, P., Sales, J., Pires de Carvalho, D., Dupuy, C., and Virion, A. (1999) Biochimie (Paris) 81, 373–380
31. Rouill, C., Tonoli, H., Bernier-Valentin, F., Rabilloud, R., Fonlupt, P., and Rousset, B. (1999) Endocrinology 140, 1990–1997
32. Bjo¨rkman, U., and Ekholm, R. (1995) EMBO J. 8, 111–116
33. Marcotte, E. M., Pellegrini, M., Thompson, M. J., Yeates, T. O., and Eisenberg, D. (1999) Nature 402, 83–86
34. Haag, R., and Dumont, J. E. (1989) Mol. Cell. Endocrinol. 8, 5294–5299
35. Marcotte, E. M., Pellegrini, M., Thompson, M. J., Yeates, T. O., and Eisenberg, D. (1999) Nature 402, 86–90
36. van Hoek, L., Van Sande, J., Ketelhant-Balasse, P., Schell-Frederick, E., and Dumont, J. E. (1982) Mol. Cell. Endocrinol. 26, 31–40
37. Björkman, U., and Ekholm, R. (1995) Mol. Cell. Endocrinol. 111, 99–107
38. Riou, C., Tonoli, H., Bernier-Valentin, F., Rabilloud, R., Fonlupt, P., and Rousset, B. (1999) Endocrinology 140, 1990–1997
39. Niepomniszcze, H., Targovnik, H. M., Gluzman, B. E., and Curutchet, P. (1987) J. Clin. Endocrinol. Metab. 62, 344–348
40. de Vlinder, J. J. M., and Vulsma, T. (1996) in Werner and Ingbar's The Thyroid (Braverman, L. E., and Utiger, R. D., eds) pp. 479–755, Lippincott Co., Philadelphia, PA
41. Corvilaïn, B., Collyn, L., Van Sande, J., and Dumont, J. E. (2000) Am. J. Physiol. Endocrinol. Metab. 278, E692–E699
42. Corvilaïn, B., Collyn, L., Van Sande, J., and Dumont, J. E. (2000) Am. J. Physiol. Endocrinol. Metab. 278, E692–E699
43. Raspe, E., and Dumont, J. E. (1995) Endocrinology 136, 965–973
44. Demeester-Mirkine, N., Van Sande, J., Dor, P., Heimann, R., Cochaux, P., and Dumont, J. E. (1984) Clin. Endocrinol. 20, 473–479
45. Savonnet, V., Maenhaut, C., Miot, F., and Pirson, I. (1997) Anal. Biochem. 247, 165–167