Hydrogen peroxide is the final electron acceptor for the biosynthesis of thyroid hormone catalyzed by thyroperoxidase at the apical surface of thyrocytes. Pig and human thyroid plasma membrane contain a Ca\textsuperscript{2+}-dependent NAD(P)H oxidase that generates H\textsubscript{2}O\textsubscript{2} by transferring electrons from NAD(P)H to molecular oxygen. We purified from pig thyroid plasma membrane a flavoprotein which constitutes the main, if not the sole, component of the thyroid NAD(P)H oxidase. Microsequences permitted the cloning of porcine and human full-length cDNAs encoding, respectively, 1207- and 1210-amino acid proteins with a predicted molecular mass of 138 kDa (p138\textsubscript{Tox}). Human and porcine p138\textsubscript{Tox} have 86.7% identity. The strongest similarity was to a predicted polypeptide encoded by a \textit{Caenorhabditis} cDNA and with rbohA, a protein involved in the \textit{Arabidopsis} NADPH oxidase. p138\textsubscript{Tox} shows also similarity to the p65\textsubscript{Mox} and to the gp91\textsubscript{Phox} in their C-terminal region and have consensus sequences for FAD- and NADPH-binding sites. Compared with gp91\textsubscript{Phox}, p138\textsubscript{Tox} shows an extended N-terminal containing two EF-hand motifs that may account for its calcium-dependent activity, whereas three of four sequences implicated in the interaction of gp91\textsubscript{Phox} with the p47\textsubscript{Phox} cytosolic factor are absent in p138\textsubscript{Tox}. The expression of porcine p138\textsubscript{Tox} mRNA analyzed by Northern blot is specific of thyroid tissue and induced by cyclic AMP showing that p138\textsubscript{Tox} is a differentiation marker of thyrocytes. The gene of human p138\textsubscript{Tox} has been localized on chromosome 15q15.

The synthesis of thyroid hormone is catalyzed by thyroperoxidase (TPO)\textsuperscript{1} in the presence of H\textsubscript{2}O\textsubscript{2} (1) on the apical membrane of the thyroid follicular cells (2). The thyroid H\textsubscript{2}O\textsubscript{2} generator is found in the apical plasma membrane of rat and pig open follicles (3, 4), and is an NAD(P)H oxidase in the pig (5–7) and human (8) thyroid, as proposed by Bjorkman \textit{et al.} (3). The thyroid NAD(P)H oxidase requires micromolar concentrations of calcium to acquire a functional conformation and to generate H\textsubscript{2}O\textsubscript{2} (7, 9). Thyrotropin (TSH) induces the expression of thyroid NAD(P)H oxidase through a cAMP-dependent pathway in the dog (10) and pig (11) thyrocytes, and transforming growth factor \(\beta\) counteracts the effect of TSH on pig cells (8). Electron transfer from NAD(P)H to molecular oxygen involves a membrane-bound flavoprotein (12) of unknown structure. Two flavoproteins involved in a mammalian NAD(P)H oxidase system have been cloned to date. One is the glyosylated flavoprotein gp91\textsubscript{Phox} involved in the respiratory burst oxidase, also called “phagocyte oxidase” (for a review, see Ref. 13). The second is the p65\textsubscript{Mox}, which participates in the activity of the “mitogenic oxidase” found in vascular smooth muscle cells (14). Both require the small membrane subunit p22\textsubscript{Phox} for their activity (13, 15). A functional NAD(P)H oxidase, generating H\textsubscript{2}O\textsubscript{2}, has been solubilized by treating pig thyroid plasma membranes with detergents at a high salt concentration (16). The flavoprotein became unable to reduce molecular oxygen after the first step of its purification by octyl-Sepharose chromatography, suggesting that it requires one or more additional component(s) to generate H\textsubscript{2}O\textsubscript{2} (16). However, the partially purified flavoprotein is still able to catalyze the NADPH-dependent reduction of the electron scavengers nitroblue tetrazolium and potassium ferricyanide (K\textsubscript{3}Fe(CN)\textsubscript{6}). We used this property to detect and quantify the flavoprotein activity during its purification (16). We have now purified the thyroid oxidase flavoprotein as a first step in defining the molecular structure of the enzyme. Peptide microsequences were used to design a gene-specific primer for rapid amplification of the 3’ cDNA ends (3’-RACE). RACE by PCR provided full-length porcine and human cDNAs encoding a protein called p138\textsubscript{Tox}, which comprises 1207 (porcine) and 1210 (human) amino acids. p138\textsubscript{Tox} is similar to a predicted 1506-amino acids protein encoded by a \textit{Caenorhabditis elegans} gene and with other flavoproteins involved in plant and mammalian NADPH oxidases. The p138\textsubscript{Tox} contains two Ca\textsuperscript{2+}-binding motifs (EF-hand) which could be involved in the control of the thyroid NADPH oxidase by Ca\textsuperscript{2+}. We also find that p138\textsubscript{Tox} is a specific marker of thyrocyte differentiation. Finally, the gene of human p138\textsubscript{Tox} was located by FISH on chromosome 15q15.

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\textsuperscript{1} The abbreviations used are: TPO, thyroperoxidase; TSH, thyrotropin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; FISH, fluorescence in situ hybridization; PAGE, polyacrylamide gel electrophoresis; kb, kilobase; bp, base pair; PAO, phe-nylarsine oxide.
Flavoproteins bound to the FAD-agarose and eluted with 50 mM sodium phosphate, pH 7.2, containing 2 mM MgCl₂, 0.25 M sucrose, 1 mM NaN₃, 0.5 mM CaCl₂, 0.4 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM KCl, and 0.2% Triton X-100 (buffer A). Human thyroid Marathon-Ready cDNAs and Advantage 2 Taq Polymerase Mix were from CLONTECH (Palo Alto, CA). Restriction enzymes and T4 DNA ligase were from Ozyme (Montigny-le-Bretonneux, France). Endoprotease Lys-C was from Roche Molecular Biochemicals (Meylan, France).

**FAD-Agarose Chromatography—**NADPH oxidase was solubilized from pig thyroid plasma membrane, and the flavoprotein partially purified by chromatography on octyl-Sepharose CL 4B (16) except that FAD was omitted from the washing buffer containing CHAPS and from the elution buffer containing 1% Triton X-100. Batches of eluted protein were stirred overnight at 4 °C with 350 µl of FAD-agarose equilibrated with 50 mM sodium phosphate, pH 7.2, containing 2 mM MgCl₂, 0.25 mM sucrose, 1 mM NaN₃, 0.5 mM CaCl₂, 0.4 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM KCl, and 0.2% Triton X-100 (buffer A). 10 mM CHAPS was then added to the suspension, which was again gently stirred overnight at 4 °C. The mix was then centrifuged (2000 x g, 30 min at 4 °C). The FAD-agarose pellet was washed twice with 3 ml of buffer B (buffer A plus 10 mM CHAPS) and once with 1.5 ml of the same buffer and suspended in SDS-gel sample buffer (1.7% final) (17). The suspension was mixed gently overnight at room temperature and centrifuged. Proteins eluted by SDS-gel sample buffer were recovered in the supernatant.

**Proteolytic Cleavage and Amino Acid Sequencing—**Analytical SDS-PAGE (8% acrylamide) was performed essentially as described by Laemmli (17). Gels were stained with 0.003% Amido Black 45% methanol, 10% acetic acid and then extensively washed in distilled water. The band of interest (3.5 µg of protein) was removed from the gel, cut into small pieces, dried under vacuum, and incubated in 50 mM Tris phase high performance liquid chromatography column (2.1 mm). Peptides were eluted with a 2–35% (v/v) linear gradient of 0.1% trifluoroacetic acid in acetonitrile over 90 min at a flow rate of 200 µl/min. The major peaks were sequenced.

**RACE by PCR—**RACE was performed using the SMART RACE cDNA amplification kit (CLONTECH, Palo Alto, CA) according to the protocol of the supplier with 1 µg total mRNA from porcine thyrocytes that had been cultivated for 4 days with 10 µM forskolin or with human thyroid Marathon-Ready cDNAs. PCR products were cloned in pCR-XL-TOPO (Invitrogen, Inc.) for sequencing. Sequencing was performed three times in the 5' to 3' direction and three times in the reverse direction.

**Primary Culture of Pig Thyrocytes—**The porcine thyrocytes (2 x 10⁶ cells/ml) were isolated by discontinuous trypsinization (18) and cultivated at 37 °C in a 5% CO₂, 95% air, water-saturated atmosphere as reconstituted follicles in suspension in 10-cm diameter untreated plastic Petri dishes (Falcon, Oxnard, CA). The cells were grown for 4 days in Eagle's minimal essential medium (pH 7.4) containing 10% (v/v) calf serum, penicillin (200 units/ml), and streptomycin sulfate (50 µg/ml), with or without 10 µM forskolin.

**RNA Isolation and Northern Blot Analysis—**The method of Chomczynski and Sacchi (19) was used to extract RNA from all cells and porcine tissues. For Northern blot analysis, total RNA (20 µg) was denatured and electrophoresed in a 1% agarose gel containing formaldehyde. Denatured RNAs were transferred by diffusion blotting onto a nylon membrane (Stratagene, La Jolla, CA) using 20 x SSC (1 x SSC = 0.15 M NaCl, 15 mM sodium citrate). The membrane was first incubated in 0.25 M sodium phosphate buffer (pH 6.8) containing 1 mM EDTA, 7% SDS, 10 mg/ml bovine serum albumin for 4 h at 65 °C and then was hybridized overnight at 65 °C with the heat-denatured probe cDNA. The cDNA probe was α-32P-labeled by random priming extension. The membrane was washed three times for 20 min in 2 x SSC, 0.1% SDS at 65 °C and then was autoradiographed at ~80 °C using Hyperfilm MP (Amersham Pharmacia Biotech). The cDNA probe used was the porcine 5'-RACE cDNA EcoR1 insert (4 kb) of pCR-XL-TOPO. The sense and antisense 5'-RACE PCR primers (Eurogentec Bel SA) were 5'-CCCCACACACACTTCATCTGGCACAGCCTGC-3' and 5'-TGGCAGGCTCAGGACATCTTCTTCC-3'.

**Fluorescent in Situ Hybridization—**The probe used to locate the p138Tos gene was the pCR-XL-TOPO vector containing the human 4-kb product of the 5'-RACE. The probe preparation and FISH conditions were essentially as described previously (20). Labeled probe (40 ng) with competitor (120 ng of human placental DNA) were denatured and placed directly on the slides without incubation at 37 °C. The signal was detected as described by Valent et al. (21).
Procedures”) with endoproteinase Lys-C generated two peptides, peptide A: AVVPPPRLYTEALQEK, and peptide B: X(V)QLIN(R/P)QDQ(T)HFVHHYEN(P), whose sequences were not found in protein data bases. Peptide B was used to design a gene-specific primer for the 3′-RACE. We carried out 5′-RACE and then amplified porcine and human full-length cDNAs by reverse transcriptase-PCR. These were cloned and sequenced. The porcine cDNA (GenBank™ accession number AF181973) is 4996-bp long, and the human cDNA (GenBank™ accession number AF181972) has 5160 bp; they are 78.1% identical (93.5% in coding region). The open reading frame (porcine: 3621 bp; human: 3630 bp) encodes a predicted protein.
with a theoretical molecular mass of 138 kDa. Kosak sequences (AG/CCATGG) were present at the translation start codon (porcine: CCACCATGG; human: CTACCATGG). The predicted protein, named p138Tox for “p138 thyroid-oxidase”, contains peptides A and B, confirming the cloning of the purified 180-kDa flavoprotein.

**General Organization of p138Tox**—Human and porcine p138Tox are 86.7% identical and contain 1210 and 1207 amino acids, respectively. p138Tox contained three main domains (Fig. 2A). The N-terminal region had similarities to peroxidases. Residues 49–206 of the human p138Tox are 27% identical to a region of pig TPO that excludes the heme-linked proximal and distal histidines. There are four putative sites of N-glycosylation on the human p138Tox and three on the pig protein (Figs. 2A and 3A), all in a predicted extracellular N-terminal domain. The difference between the 138-kDa theoretical molecular mass and the 180-kDa apparent mass of the flavoprotein could reflect the presence of complex sugars at these N-glycosylation sites. A median domain is similar to the Ca\(^{2+}\) -binding proteins containing Ca\(^{2+}\) -EF-hand motifs. The C-terminal domain is similar to the Ca\(^{2+}\) -binding sites of gp91Phox and p67Phox, the large subunits of phagocyte oxidase.

**Similarity to Non-mammalian Proteins**—The overall sequence of p138Tox is very similar to that of a predicted protein from *C. elegans* (GenBank™ accession number AF043697). In addition to its glycosylated N terminus being similar to peroxidases, the *Caenorhabditis* protein has two EF-hand motifs in the middle of the primary structure (Fig. 2A) which are conserved in p138Tox \(^{149}\)DKDGNYLFSREF\(^{206}\) and \(^{539}\)DLENG\(^{542}\)FLSKDEF in human sequence). These Ca\(^{2+}\) -binding sites could be involved in the direct activation of the thyroid H\(_2\)O\(_2\) generator by Ca\(^{2+}\). The flavoprotein p138Tox is also similar to rbohA, a homolog of gp91Phox involved in the respiratory burst oxidase of *Arabidopsis thaliana*, which also contains two Ca\(^{2+}\) -binding EF-hand motifs in its hydrophilic N-terminal domain.

Hydropathicity plots obtained by the method of Kyte and Doolittle (23) showed similar distributions of seven hydrophobic stretches in *Caenorhabditis* protein and in p138Tox, indicating that the proteins share a well conserved tertiary structure (Fig. 2B). The first five hydrophobic stretches are probably membrane-spanning domains, according to the TMpred program (24), with the glycosylated N-terminal outside the cell and the EF-hand motifs logically located inside. However, p138Tox is also a truncated mammalian version of the *Caenorhabditis* protein, which has an additional 331 amino acids at its N terminus. *Arabidopsis* rbohA has a similar, but even shorter, N terminus (Fig. 2A), so that the first membrane-spanning domain is absent as well as the peroxidase-like glycosylated domain.

**Similarities to Mammalian Oxidases**—The C-terminal regions of p138Tox rbohA, and the *Caenorhabditis* protein are very similar to the gp91Phox and p67Phox, with a similar distribution of six hydrophobic stretches. An isoalloxazone FAD-binding motif of gp91Phox \(^{333}\)HPFTLTS\(^{344}\) is conserved in human (978–984) and porcine (975–981) p138Tox as shown (Fig. 3B) by the multiple alignment of human and porcine p138Tox sequences with human gp91Phox sequence made with the CLUSTALW program (25). The ribose \(^{410}\)GIGVTPF\(^{416}\) and adenine \(^{514}\)FXCGP\(^{539}\) NADPH-binding motifs of gp91Phox are also found in the C-terminal region of human (1048–1054) and porcine (1045–1051) p138Tox and should lie in a cytosolic domain. The four histidine residues, which are probably involved in heme binding in gp91Phox are conserved in p138Tox.

**Structural Differences between gp91Phox and p138Tox**—Whereas the FAD and NADPH binding sites of gp91Phox are clearly present in p138Tox, alignment of the two sequences indicates some differences. For example, the N-glycosylation sites of p65Phox and gp91Phox are absent from the p138Tox, rbohA, and *Caenorhabditis* protein C-terminal regions. p138Tox also differs from gp91Phox in some other sequence features, which could be relevant to the different specific mechanisms involved in their activation. Previous biochemical studies (7, 9) showed that micromolar concentrations of Ca\(^{2+}\) were necessary and sufficient to cause complete, reversible activation of thyroid oxidase, suggesting that interaction of the flavoprotein with cytotoxic factors such as p47Phox and p67Phox is not required, unlike the respiratory burst oxidase. Indeed, three of the four motifs involved in p47Phox binding to gp91Phox subunit are not present in p138Tox (Fig. 3B). Furthermore, the tervalent arsenoxide phenylarsine oxide (PAO), a hydrophobic molecule that reacts with two vicinal thiol groups in proteins, alters the structure of the porcine thyroid oxidase so that electron transfer occurs more slowly but without Ca\(^{2+}\) (26). The porcine and human p138Tox contain only two adjacent cysteine residues (Fig. 3A), which are located in the first predicted membrane-spanning domain (268C and 269C), just before the intracellular segment containing the two EF-hand motifs. They are probably the binding site of PAO, reinforcing the idea that this region, absent from gp91Phox, is crucial for the control of the thyroid NAD(P)H oxidase activity by Ca\(^{2+}\). PAO also affects the respiratory burst oxidase, but in a different manner, because it essentially prevents the human (27) and bovine (28) oxidase activation by cytotoxic factors through its binding to gp91Phox. This particular effect could result from the binding of PAO to the only two adjacent cysteine residues of gp91Phox, located just before the first peptide involved in activation by p47Phox \(^{86}\)CCSTRVRQR\(^{94}\), a sequence which is absent from p138Tox (Fig. 3B).

**Tissue Distribution of the p138Tox mRNA**—Fig. 4A shows that the 5-kb p138Tox mRNA was detected by Northern blot only in thyroid tissue. This strongly supports the idea that the cloned flavoprotein actually participates in the specific function...
of thyrocytes, the synthesis of thyroid hormones, as an essential component the H₂O₂ generator associated with TPO. The thyroid-specific expression of the p138 mRNA also justifies the designation of the NADPH oxidase as “thyroid oxidase” or “tox.”

Flavoprotein p138^tox as a Marker of Thyrocyte Differentiation—Fig. 4B shows that porcine thyrocytes cultured without forskolin do not contain the 5-kb p138^tox mRNA, whereas its concentration is maintained, or even enhanced, by cyclic AMP. These data are in good agreement with those of Raspe and concentration is maintained, or even enhanced, by cyclic AMP.

We have demonstrated that the thyroid NAD(P)H oxidase is different from other known mammalian NADPH oxidases. Its flavoprotein is not only a new homolog of gp91^phox and p65^mox, but it also has specific structural features that can account for its biochemical properties. Among them is the presence of two calcium binding EF-hand motifs, which have only been found in non-mammalian NADPH oxidases to date. Its tissue specificity and cAMP-regulated expression makes p138^tox a new marker of thyrocytes, which could be relevant in thyroid disorders.

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