Activation of Dual Oxidases Duox1 and Duox2

DIFFERENTIAL REGULATION MEDIATED BY cAMP-DEPENDENT PROTEIN KINASE AND PROTEIN KINASE C-DEPENDENT PHOSPHORYLATION∗†

Received for publication, September 5, 2008, and in revised form, January 12, 2009 Published, JBC Papers in Press, January 14, 2009, DOI 10.1074/jbc.M806893200

Sabrina Rigutto,1,2 Candice Hoste,1 Helmut Grasberger,3,5 Milutin Milenkovic1, David Communi,4 Jacques E. Dumont, Bernard Corvilain,5 Françoise Miot1, and Xavier De Deken

From the 1Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire and the 5Department of Endocrinology, Hôpital Erasme, Université Libre de Bruxelles, Campus Erasme, 1070 Brussels, Belgium and the 4Department of Medicine, University of Chicago, Chicago, Illinois 60637

Dual oxidases were initially identified as NADPH oxidases producing H$_2$O$_2$ necessary for thyroid hormone biosynthesis. The crucial role of Duox2 has been demonstrated in patients suffering from partial iodide organification defect caused by biallelic mutations in the DUOX2 gene. However, the Duox1 function in thyroid remains elusive. We optimized a functional assay by co-expressing Duox1 or Duox2 with their respective maturation factors, DuoxA1 and DuoxA2, to compare their intrinsic enzymatic activities under stimulation of the major signaling pathways active in the thyroid in relation to their membrane expression. We showed that basal activity of both Duox isoenzymes depends on calcium and functional EF-hand motifs. However, the two oxidases are differentially regulated by activation of intracellular signaling cascades. Duox1 but not Duox2 activity is stimulated by forskolin (EC$_{50}$ = 0.1 µM) via protein kinase A-mediated Duox1 phosphorylation on serine 955. In contrast, phorbol esters induce Duox2 phosphorylation via protein kinase C activation associated with high H$_2$O$_2$ generation (phorbol 12-myristate 13-acetate EC$_{50}$ = 0.8 mM). These results were confirmed in human thyroid cells, suggesting that Duox1 is also involved in thyroid hormoneogenesis. Our data provide, for the first time, detailed insights into the mechanisms controlling the activation of Duox1–2 proteins and reveal additional phosphorylation-mediated regulation.

Dual oxidases (Duox1 and Duox2) belong to the family of NADPH oxidases (Nox), which is composed of five additional enzymes: Nox1–5 (1–3). These transmembrane proteins are characterized by a COOH-terminal NADPH oxidase catalytic core responsible for reactive oxygen species synthesis. The best characterized NADPH oxidase Nox2 is involved in the leukocyte respiratory burst and activated by invading pathogens (4). The mechanisms controlling the Nox-mediated reactive oxygen species production are multiple and complex. The activation of Nox1 (5, 6), Nox2 (3, 7), and Nox3 (8, 9) requires the coordinated assembly of several subunits: the association with the transmembrane protein p22$^{phox}$ and the recruitment of three cytosolic proteins, the small G protein Rac, p47$^{phox}$ (or NOXO1), and p67$^{phox}$ (or NOXA1).

Duox1 and Duox2 isoenzymes are large members of the Nox/Duox family. In addition to the catalytic core, Duox1 and Duox2 proteins are NH$_2$-terminally extended by an extracellular peroxidase-like domain followed by a membrane-spanning segment and an intracellular domain comprising two canonical EF-hand motifs (2). Duox1 and Duox2 are the sole proteins directly generating H$_2$O$_2$ (10) outside the cells, whereas small Nox homologues are mostly superoxide generators (3). Duox isoforms do not need to be associated with cytosolic factors to be active but undergo a critical maturation process necessary to acquire their active conformation at the apical cell surface of the thyrocytes (11). The immature nonfunctional form (180 kDa) is not properly glycosylated and maintained in the endoplasmic reticulum compartment. Only the co-expression of the Duox maturation factors (DuoxA1 and DuoxA2) allows functional reconstitution. They are N-glycosylated proteins permitting the endoplasmic reticulum exit of properly folded Duox enzymes (12, 13). The DUOXA genes are localized near their respective DUOX gene in a head to head orientation on chromosome 15 and co-expressed with their Duox counterpart in the same tissues (12).

Dual oxidases expressed at the apical side of surface epithelia exposed to microorganisms, like the airways or the digestive tract, are supposed to function as components of the innate host defense system (14–16). However, Duox1 and Duox2 isoenzymes were initially identified as H$_2$O$_2$-generating thyroid oxidases (1, 2). The main function of the thyroid is the uptake and concentration of iodide from the bloodstream to synthesize thyroid hormones (T3 and T4) in the follicular lumen (17). H$_2$O$_2$ produced at the apical pole of the thyrocyte is utilized by thyroperoxidase as an electron acceptor to oxidize iodide, covalently link oxidized iodide to tyrosines of thyroglobulin,
and couple iodinated tyrosyl residues to form protein-bound iodothyronines (T3 and T4) (18). Under physiological iodide supply, hormonogenesis is rate-limited by the availability of hydrogen peroxide (19). Patients presenting iodide organification defect caused by mutations in their DUOX gene suffer from transient or permanent hypothyroidism depending on the mono- or bi-allelic character of the mutation, demonstrating the crucial function of Duox2 in thyroid hormone synthesis (20–25). However, the physiological meaning of the co-existence of the two dual oxidases and their respective maturation factors in the thyroid tissue remains an open question.

In this study, we analyzed the mechanisms of activation of Duox1 and Duox2 using Duox/DuoxA co-transfected cells stimulated by agonists known to regulate the thyroid metabolism (26, 27). Our results indicate that Duox1 and Duox2 activities are mainly calcium-dependent NADPH oxidases. Moreover, additional mechanisms governing their intrinsic activity are different; Duox1 is positively regulated by the cAMP-dependent protein kinase A (PKA)6 cascade, whereas Duox2 is highly induced by activation of protein kinase C (PKC) with very low concentrations of PMA.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Mutagenesis**—The wild type untagged versions of human Duox1 (accession number AF230495) and Duox2 (accession number AF230496) cDNA were cloned from ATG to stop codon into the vector pcDNA3 (Invitrogen). The NH2-terminal hemagglutinin epitope-tagged human Duox2 (HA-Duox2-pcDNA3.1) and the COOH-terminal c-Myc epitope-tagged human DuoxA1 and DuoxA2 (DuoxA1-Myc-pcDNA3.1 and DuoxA2-Myc-pcDNA3.1) were described elsewhere (12). We added an additional Rho tag to Duox1 to be able to distinguish it from HA-Duox2 in future experiments. The Duox1 native signal peptide was replaced by the TSH receptor signal peptide to facilitate the cloning step. The Rho-HA-Duox1-pcDNA3 construct was generated as follows. The 23 first amino acids (residue 1 corresponding to the initiation methionine) of the human Duox1 protein (accession number AAF73921) were replaced by the signal peptide of the human TSH receptor (MPRADLQVLLLDLPRDLGG) (accession number CA02195) and the first 19 residues of the bovine rhodopsin (Rho) (accession number P02699) (MNGTEGPNFYVPFSNKTGVV; two putative glycosylation sites are underlined) (28). The cDNA corresponding to the NH2-terminal HA-tagged Duox1 protein (24–1551 amino acids) was cloned just downstream of the Rho tag by insertion of an EcoRI site. The presence of the TSH receptor signal peptide, Rho, and HA tags at the NH2 terminus of Duox1 did not affect its expression nor its peroxide generating activity (supplemental Fig. S1). Furthermore, the extra N-linked sugars present on the Rho tag have been very useful to separate the two glycosylated forms of Duox1 for mass spectrometry analysis, because it is mainly the mature highly glycosylated form of Duox1 that is phosphorylated upon stimulation.

Mutations in Duox1–2 were introduced by directed mutagenesis with the QuickChange system (Stratagene, La Jolla, CA) (primers are described in supplemental Table S1). All of the constructs were verified by Big Dye Terminator cycle sequencing on an automated ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA).

**Cell Culture and Transfection**—Cos-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Invitrogen), 2% streptomycin-penicillin, 1% fungizone, and 1% sodium pyruvate. For H2O2 assay, adherent cells at 50–60% confluence were transfected in 6-well plates using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocol (ratio: 1 μg of DNA for 3 μl of FuGENE 6) with 500 ng of Rho-HA-Duox1-pcDNA3 and 500 ng of DuoxA1-Myc-pcDNA3.1 or with 500 ng of HA-Duox2-pcDNA3.1 and 500 ng of DuoxA2-Myc-pcDNA3.1. Under optimal conditions, the transfection efficiency reached 20–30% of cells expressing Duox proteins at the cell surface detected by FACS. For immunoprecipitation experiments, the cells seeded in 10-cm-diameter dishes were transfected with 8 μg of DNA and 24 μl of FuGENE 6.

**Human Thyroid Primary Culture**—Human thyroid tissue was obtained from patients undergoing partial or total thyroidectomy for resection of solitary cold nodules or multinodular goiters. Only healthy, normal-looking, non-nodular tissue was used within 30 min after surgical removal. Thyrocytes in primary culture obtained from follicles isolated by collagenase digestion and differential centrifugation were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12/MCDB104 (2:1:1 medium (Invitrogen) with 1% sodium pyruvate, 40 μg/ml ascorbic acid, 5 μg/ml insulin, 2% streptomyacin-penicillin, and 1% fungizone (29, 30). Thyrocytes were seeded 5 days before the assay. The protocol has been approved by the hospital ethics committee.

**H2O2 Measurement and Flow Immunocytometry Analysis**—Production of H2O2 was determined by the sensitive fluorimetric method of Bénard and Brault (31) slightly modified as previously described (11). The peroxide released from transfected cells (Duox1/DuoxA1 or Duox2/DuoxA2) into Krebs-Ringer-Hepes medium was accumulated in the presence of stimulating agents for 2.5 h at 37 °C. After removing the medium, cell surface expression of Duox1–2 proteins was measured by flow cytometry (FACS). Briefly, the cells detached with phosphate-buffered saline EDTA/EGTA (5 mM) were incubated sequentially with anti-HA antibody (clone 3F10; Roche Applied Science) and fluorescein-conjugated anti-rat IgG, both diluted 1/100 in phosphate-buffered saline, 0.1% bovine serum albumin. Propidium iodide (5 μg/ml) staining in the second incubation step was used to exclude damaged cells from subsequent analysis. Fluorescence was analyzed using cell sorting (FACS; Becton Dickinson, Erembodegem, Belgium) counting 20,000 events/sample. Relative protein expression was determined by calculating the differences in total fluorescence intensity (Arbitrary Unit) between the samples and an equal-sized population of control cells expressing only Duox1 or Duox2 constructs without their respective maturation factors. Without the latter, no cell surface expression of Duox could be detected (11). H2O2 production was normalized to cell surface

---

6 The abbreviations used are: PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; WT, wild type; HA, hemagglutinin; TSH, thyroid-stimulating hormone; FACS, fluorescence-activated cell sorter; Fsk, forskolin; 6-MB-cAMP, N6-monobutyryladenosine-3’,5’-cyclic monophosphate.

---

**Activation Mechanisms of Duox1–2 Isoenzymes**
expression of each construct and reported as pg of H₂O₂/FACS. For cultures of human thyrocytes, H₂O₂ released over 90 min in the presence of various agents was normalized to total proteins extracted in Laemmli buffer and quantified by paper dye binding assay (ng of H₂O₂/µg of protein) (32).

Immunoprecipitation and Western Blot Analysis—Proteins were extracted in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.5% Nonidet P-40, 120 mM β-mercaptoethanol, 100 mM NaF, 2 mM EDTA, pH 8.0, 50 mM okadaic acid, 1 mM vanadate) supplemented with a mixture of protease inhibitors (Complete; Roche Applied Science) for 1 h at 4 °C. The lysate was centrifuged 15 min at 10,000 rpm, and the supernatant was pre-cleared with Sepharose beads (GE Healthcare). Duox1 complexes were immunoprecipitated with anti-Duox antibody (1/100) conjugated to Sepharose beads (2) and Duox2 proteins with monoclonal anti-HA antibody precoated on agarose beads (Clone HA-7; Sigma-Aldrich). Proteins of the immunoprecipitated were separated by SDS/PAGE and transferred to nitrocellulose as previously described (2). Phosphorylated Duox proteins were detected using a rabbit polyclonal antibody raised against phospho-(Ser/Thr) PKA substrate (1/1,000; Cell Signaling, Danvers, MA), which is directed to the phospho motif RX(S/T). Fluorescent secondary antibodies (1/10,000; IRDye 800 anti-rabbit from LI-COR, Lincoln, NE) were used for image acquisition and quantification with the Odyssey infrared imaging system (LI-COR). The membrane was stripped and immunoblotted with the anti-Duox antibody (1/16,000) and fluorescent secondary antibodies (1/10,000; IRDye 680 anti-rabbit) to quantify total Duox proteins.

Radioactive Phosphorus Incorporation—Cells maintained 24 h in serum-free phosphate-depleted medium were incubated 2 h with 500 µCi (Cos-7) or 1mCi (thyrocytes) of [³²P]orthophosphate. The proteins were prepared as described above, and phosphorylated proteins were detected and quantified with a Storage Phosphor Screen (GE Healthcare) scanned and no H₂O₂ was detected in cells expressing Duox or DuoxA (Fig. 1A). The adenylate cyclase agonist, forskolin (Fsk), raised the amount of H₂O₂ generated by Duox1 but not by Duox2 with a specific activity of both Duox enzymes was comparable, and no H₂O₂ was detected in cells expressing Duox or DuoxA alone (Fig. 1A). 3) We verified that the addition of the tags did not modify the expression and activity of Duox1–2 proteins (supplemental Fig. S1). In all experiments, the specific activity of the Duox enzymes is represented as the amount of H₂O₂ produced normalized to their surface expression (pg H₂O₂/FACS).

In Vitro PKA Phosphorylation Assay—Cos-7 Duox1/DuoxA1 transfected cells were cultured 24 h without serum. The proteins were prepared in lysis buffer, and Duox1 complexes were immunoprecipitated overnight with monoclonal anti-HA antibody precoated on agarose beads as described above. Immunoprecipitated proteins were incubated for 10 min at 37 °C in a 50-µl final volume that contained 20 mM Hepes, pH 7.4, 0.1 mM dithiothreitol, 10 mM MgCl₂, 0.1 mM [γ-³²P]ATP (5 µCi/tube), and 100 ng of purified catalytic subunit of protein kinase A (Calbiochem, Gibbstown, NJ). After SDS/PAGE and Western blotting, radioactive signals were quantified with Typhoon Trio+ (GE Healthcare). Total Duox proteins for each condition were measured by Odyssey infrared imaging system coupled with the polyclonal anti-Duox antibody.

Activation Mechanisms of Duox1−2 Isoenzymes

RESULTS

DuoxA-based Functional Assay to Study Duox-mediated H₂O₂ Generation—Heterologous systems combining DuoxA expression have already been successfully used to characterize Duox activity of human Duox2 natural mutants (13) and to reconstitute a functional H₂O₂-generating system in a lung cancer cell line (34). The originality of our study is to analyze the specific activity of Duox1 and Duox2 isoenzymes by normalizing the hydrogen peroxide production to the cell surface expression of the respective proteins. Insertion of an HA tag in the Duox1−2 ectodomain provides an effective means to reliably estimate Duox membrane expression and compare the activities of the two enzymes. We first validated our heterologous system: 1) Measurement of H₂O₂ produced after 1 µM ionomycin stimulation of cells transfected with Duox/DuoxA (constant DuoxA quantity, 50 ng) was proportional to the quantity of transfected Duox plasmids (25–500 ng) with a plateau reached at 250–500 ng of Duox DNA probably caused by a limited amount of DuoxA (Fig. 1A). 2) We observed a linear relationship between Duox-mediated H₂O₂ generation and the membrane expression of Duox as measured by FACS (Fig. 1B). The specific activity of both Duox enzymes was comparable, and no H₂O₂ was detected in cells expressing Duox or DuoxA alone (Fig. 1A). 3) We verified that the addition of the tags did not modify the expression and activity of Duox1–2 proteins (supplemental Fig. S1). In all experiments, the specific activity of the Duox enzymes is represented as the amount of H₂O₂ produced normalized to their surface expression (pg H₂O₂/FACS).

In humans, the thyroid metabolism is under the control of the phosphatidylinositol 4,5-bisphosphate cascade and the cAMP cascade (26, 27). In our functional assay, 1 µM ionomycin increased the activity of both Duox1 and Duox2 enzymes to the same level, but raising concentrations of ionomycin from 2 to 4 µM resulted in higher activity of Duox1 than Duox2 (Fig. 2A). The adenylate cyclase agonist, forskolin (Fsk), raised the amount of H₂O₂ generated by Duox1 but not by Duox2 with a 50% effective concentration (EC₅₀) of 0.1 µM (Fig. 2B). Moreover PMA, a PKC activator, differentially regulated activities of Duox1 and Duox2 (Fig. 2, C and D). Although micromolar concentrations of PMA were needed to increase Duox1 activity with an EC₅₀ of 1.8 µM, a maximal activation of Duox2 enzyme was already observed with nanomolar PMA concentrations.
Activation Mechanisms of Duox1–2 Isoenzymes

FIGURE 1. Intrinsic activity of Duox isoenzymes in reconstituted systems. A, measurement of H$_2$O$_2$ accumulation (ng) produced by Duox1/DuoxA1 or Duox2/DuoxA2 co-transfected Cos-7 cells for 2.5 h in the presence of 1 μM ionomycin. Constant amount of DuoxA-Myc-pcDNA3.1 DNA (50 ng) was transfected with increasing DNA amounts of Rho-HA-Duox1-pcDNA3 (white) or HA-Duox2-pcDNA3.1 (black) (0–500 ng). Each measurement corresponds to a transfection experiment performed in duplicate (means ± S.D.) B, plot of H$_2$O$_2$ production against the relative Duox expression level at the cell surface. Membrane expression of Rho-HA-Duox1 (□) and HA-Duox2 (■) proteins were quantified by FACS using the anti-HA antibody (AU, arbitrary unit of fluorescein isothiocyanate fluorescence intensity). Inset, representative histograms of a FACS experiment; the gray areas represent cells transfected with the Duox construct alone. The percentage of cells expressing Duox at the cell surface is indicated for each construct.

The addition of Fsk or PMA to ionomycin in the incubation medium provoked a release of H$_2$O$_2$ corresponding to the sum of the amounts generated by the cells treated with the agents separately (Iono versus Iono+Fsk for Duox1: p < 0.01; Iono versus Iono+PMA for Duox1: p < 0.001; Iono versus Iono+PMA for Duox2: p < 0.01) (Fig. 2E). In contrast to Duox1, raising calcium concentration was not sufficient to activate Duox2 by Fsk. In all of the conditions, 1 μM dipher-nyleiodonium, a flavoprotein inhibitor, reduced the H$_2$O$_2$ level produced by the two enzymes, indicating that it derives from an NADPH oxidase (data not shown).

Regulation of Duox Activity by Calcium—Duox-mediated H$_2$O$_2$ generation is presumed to be regulated by binding of Ca$^{2+}$ to the two EF-hand motifs, located in the cytosolic portion spanning transmembrane domain 1 and 2 (13, 34, 35). Indeed, the absence of calcium ions in the H$_2$O$_2$ measurement assay abolished the Duox1–2 activity (data not shown). The other calcium-dependent NADPH oxidase, NOX5, possesses four calcium-binding sites arranged in two functional pairs (36). To study the role of the two Duox EF-hands, the crucial glutamate residue was replaced by a glutamine in the twelfth position of the EF-hand sequence. Duox2 mutants E843Q and E879Q completely lost the ability to produce H$_2$O$_2$ in basal or stimulated conditions but maintained a cell surface expression similar to the wild type (WT) protein (Fig. 3). Similarly, inactivation of one of the two EF-hands in Duox1 (E839Q and E875Q Duox1 mutants) was sufficient to inactivate Duox1, although a correct processing to the membrane occurred as for Duox2 (supplemental Fig. S2). These results strongly suggest that the two EF-hand motifs operate as one functional pair necessary for Duox activation in response to an increase of intracellular calcium concentration.

Duox1 Activity Is Positively Modulated through the CAMP Pathway—As shown in Fig. 2, Duox1-dependent H$_2$O$_2$ generation was positively controlled by Fsk, contrary to Duox2. To establish the role of the protein kinase A, N$^6$-monobutyryladenosine-3’,5’-cyclic monophosphate (6-MB-cAMP), a site-selective PKA agonist, was used. H$_2$O$_2$ produced by Duox1 was significantly increased with either 50 μM 6-MB-cAMP or 1 μM Fsk (Fig. 4A). We also co-transfected a vector encoding the α isoform of the PKA catalytic subunit with WT Duox/DuoxA constructs (37, 38). Overexpression of PKA for 48 h, verified by Western blotting (data not shown), also induced an increase of Duox1 activity. Co-expression of the PKA subunit had only a minor effect on Duox2 activity, and treatment with the 6-MB-cAMP did not increase H$_2$O$_2$ production by Duox2.

Motif scanning for PKA substrates based on the consensus sequence (R/K)2X(S/T) identifies three potential PKA phosphorylation sites in Duox1 (Ser$^{955}$, Thr$^{1007}$, and Ser$^{1217}$) (supplemental Fig. S3). We analyzed the Duox1 phosphorylation state with an anti-RXX(pS/pT) antibody that could potentially recognize phosphorylation on Ser$^{955}$ and Ser$^{1217}$ but not on Thr$^{1007}$. Under basal conditions, the mature form of Duox1 (200 kDa) was already phosphorylated in Cos-7 cells (supplemental Fig. S4). It is noteworthy that the slower migrating form of Duox1 (mature form) and the immature form were shifted to 200 and 190 kDa, respectively, instead of the wild type 190- and 180-kDa proteins. This phenomenon can be explained by the addition of extra N-linked sugars on the two putative N-glycosylation sites present in the Rho tag sequence as previously described for similar TSH receptor constructs (28). Duox1 phosphorylation was stimulated by Fsk in a time-dependent way with a maximum reached after 30 min of stimulation (supplemental Fig. S4). Increased Duox1 phosphorylation was also observed in cells treated 30 min with 6-MB-cAMP or cells over-expressing the PKA catalytic subunit (Fig. 4B). The stimulatory effect of Fsk was also observed on H$_2$O$_2$ accumulation for 30 min (data not shown). Constitutive and PKA-induced phosphorylation of Duox1 was evident, in $^{32}$P incorporation experiments, with the detection of a major radioactive band corresponding to the high molecular mass form (200 kDa) of Duox1 (supplemental Fig. S5). No increase of Duox2 phosphorylation
could be detected under Fsk stimulation by $^{32}$P incorporation experiments (data not shown).

Identification of PKA Target Sites in Duox1—To identify the phosphorylated sites by PKA, Duox1 protein was isolated from Fsk-stimulated Cos-7 cells co-transfected with Duox1/DuoxA1 and subjected to mass spectrometry analysis. After trypsin or chymotrypsin digestions, two phosphorylated peptides were isolated and microsequenced: the peptide $^{954}$ASYISQDMICP$^{967}$, which encloses the serine 955 (underlined), and the peptide $^{1214}$RRRSFRG$^{1221}$ including the serine 1217. Three peptides containing the threonine 1007 ($^{1006}$VT$^*$FQPLLFTEAHR$^{1021}$, $^{1006}$KV$^*$FQPLLFTEAHR$^{1019}$, and $^{1006}$VT$^*$FQPLLFTEAHR$^{1019}$) were also isolated but were not phosphorylated.

To address the role of these potential phosphorylated residues in Duox1 activity, we replaced them with the nonphosphorylatable amino acid, alanine. Three single mutants (S955A, T1007A, and S1217A) and one double mutant (S955A/S1217A) were constructed. Cos-7 cell surface expression of T1007A and S1217A mutants was similar to membrane expression of WT protein, whereas S955A and S955A/S1217A were less expressed than the WT Duox1 (Fig. 5A, inset). The basal activity of S955A Duox1 was severely impaired and was no longer stimulated after Fsk treatment, whereas it still responded to ionomycin and PMA (Fig. 5A). Mutant T1007A presented a slightly lower activity than the WT protein but was still positively regulated by all agonists. Interestingly, mutation S1217A generated an enzyme with increased basal activity, and the double mutant S955A/S1217A produced a similar H$_2$O$_2$ amount as WT Duox1 in basal and stimulated conditions, except for a loss of response to Fsk.

Phosphorylation state of Duox1 mutants in basal condition or after Fsk stimulation was analyzed by radioactive phosphorus incorporation (Fig. 5B). Basal phosphorylation of the 200-kDa mature form of S955A and S1217A Duox1 was highly decreased compared with the WT Duox1 but was still stimulated by Fsk. The T1007A mutant presented the same Fsk-dependent phosphorylation pattern as WT Duox1. On the other hand, the S955A/S1217A double mutant showed a very low basal phosphorylation no longer stimulated by Fsk. The same experiment was performed using the anti-phospho PKA substrate antibody. Unfortunately, this antibody was unable to recognize the Ser1217 and Thr$^{1007}$ as demonstrated by the complete absence of phosphorylation of the S955A mutant (supplemental Fig. S6). Nevertheless, the results confirmed the Fsk-mediated phosphorylation of serine 955. Direct phosphorylation of Duox1 was measured in vitro. In the presence of purified PKA, WT and T1007A Duox1 showed robust phos-
time-dependent phosphorylation of Duox2, already visible after 10 min of exposure to PMA that was prevented by Ro318220 (Fig. 6, B and C). A 30-min treatment with 1 nM PMA significantly increased \( \text{H}_2\text{O}_2 \) generation from Duox2/DuoxA2 co-transfected cells (data not shown). No increase of Duox1 phosphorylation could be observed after PMA treatment, and the inhibitor Ro318220 did not inhibit PMA-stimulated Duox1 activity (data not shown). These results suggest that physiologically PKC-\( \alpha \) or -\( \beta_1 \) isoforms participate in the phosphorylation and activation of Duox2 and not Duox1.

Regulation of \( \text{H}_2\text{O}_2 \) Generation in Human Thyroid Primary Cultures—In the functional assay, we found that Duox1 and Duox2 activities are both calcium-dependent but differentially regulated by PKA and PKC. To determine the physiological relevance of these results, we analyzed the modulation of \( \text{H}_2\text{O}_2 \) production in human thyrocytes under similar stimulation conditions. \( \text{H}_2\text{O}_2 \) measurements from primary cultures showed that basal \( \text{H}_2\text{O}_2 \) accumulation (0.31 ng of \( \text{H}_2\text{O}_2/\mu\text{g of protein} \)) was increased by ionomycin (4.08 ng of \( \text{H}_2\text{O}_2/\mu\text{g of protein} \)) and not by Fsk alone (Fig. 7A). Combining Fsk and ionomycin treatments significantly increased the \( \text{H}_2\text{O}_2 \) production to 7.16 ng of \( \text{H}_2\text{O}_2/\mu\text{g of protein} \) (Iono versus Iono+Fsk, \( p < 0.001 \)), demonstrating a permissive effect of calcium rather than an additive effect observed in the heterologous system (Fig. 2E). Ionomycin also increased the \( \text{H}_2\text{O}_2 \) amount in response to PMA to a similar extent (Iono versus Iono+PMA, \( p < 0.001 \)). The 190-kDa mature form of Duox proteins was phosphorylated in basal condition and showed increased phosphorylation level after stimulation with Fsk or PMA as estimated by \( ^{32}\text{P} \) incorporation (Fig. 7B). This Duox phosphorylation was reproduced by 1 or 10 milliunits/ml of the thyrotropin hormone (TSH), concentrations described to stimulate either the adenylyl cyclase-cAMP pathway or the phospholipase C-diacylglycerol-calcium cascade, respectively (27).

**DISCUSSION**

The biochemical function of hydrogen peroxide in thyroid hormone synthesis has been known for decades (18). A flavoprotein complex was predicted to produce directly \( \text{H}_2\text{O}_2 \) outside the cell with an activity mediated by NADPH and stimulated by calcium (10, 35, 39, 40). In 2000, the cloning of two cDNAs encoding novel calcium-dependent NADPH oxidases, Duox1 and Duox2, revealed the molecular nature of the presumed thyroid \( \text{H}_2\text{O}_2 \)-generating system (1, 2, 11). However, functional studies on dual oxidases have been conducted only recently thanks to the discovery of their maturation factors, DuoxA1 and DuoxA2, allowing correct processing of Duox1–2 isoenzymes in heterologous systems (12, 13). In this work, we improved a DuoxA-based functional assay to compare the specific activities of Duox1 and Duox2, and we uncovered mechanisms of activation that discriminate between the two oxidase activities.

Each Duox protein possesses two canonical EF-hand motifs that are involved in the main regulation step exerted by calcium (2, 13, 34, 41). Co-expression of Duox/DuoxA proteins in Cos-7 cells reconstitutes an \( \text{H}_2\text{O}_2 \) generator with an activity acutely stimulated by an elevation of the intracellular calcium concentration. We have demonstrated that intact EF-hand sequences are required to maintain functional Duox proteins. Moreover,
whereas the two dual oxidases exhibit similar activity at low ionomycin concentration (1 μM), Duox1 seems to be more efficient than Duox2 in cells treated with higher ionomycin concentrations. This effect might be explained by the differences in the amino acid sequence of the Duox1 second EF-hand motif.

Recent reports have shown that, in addition to calcium, the Nox5 enzymatic activity is also regulated through PKC-mediated phosphorylations on serine and threonine residues enhancing its sensitivity to calcium (42). Using radioactive phosphorus incorporation and anti-phospho-PKA substrate antibody, we demonstrated that Duox1 is also a phosphoprotein. The enzyme is constitutively phosphorylated, and activation of the PKA pathway increases its phosphorylation state as well as its activity. Immature Duox1–2 proteins generate essentially superoxide inside the cell and become H₂O₂ generators only at the cell surface (41). In transfected cells, in vivo 32P incorporation shows that mainly the mature form of Duox1 is phosphorylated in basal and stimulated conditions, even if the immature form of Duox1 is more abundant (Fig. 5B). These data suggest that, in intact cells, Duox isoenzymes might undergo conformational changes during their processing from the endoplasmic reticulum to the plasma membrane modifying the accessibility of key residues to the kinase. In in vitro PKA phosphorylation experiments, these amino acids could be artificially demasked by detergents showing elevated phosphorylation intensity for the immature form of Duox1 (Fig. 5C).

Mass spectrometry analyses revealed Duox1 phosphopeptides containing the residues Ser⁹⁵⁵ and Ser¹²¹⁷ in co-transfected Cos-7 cells in response to Fsk. Systematic mutations of potential PKA-mediated phosphorylation sites have identified Ser⁹⁵⁵ and Ser¹²¹⁷ as major residues responsible for the basal phosphorylation state of Duox1. These two serines are conserved among human, dog, pig, mouse, and rat species. However, rendering these amino acids non-phosphorylatable reveals distinct effects on Duox1 specific activity. The S1217A variant presents higher constitutive activity than the WT enzyme and still responds to cAMP cascade.
activation, whereas the S955A substitution decreases basal and Fsk-stimulated Duox1 activity. These results demonstrate that serine 955 is the crucial residue positively regulating Duox1 activity through PKA-mediated phosphorylation. It could increase the sensitivity of Duox1 to lower level of intracellular calcium as has been demonstrated for Nox5 enzyme (42). The hyperactivity of the mutant S1217A was more surprising and suggests an inhibitory effect on the overall Duox1 activity driven by its phosphorylation state. Our hypothesis is that in resting cells, constitutive phosphorylation on Ser1217 restrains the enzyme to limit peroxide generation, whereas PKA-mediated phosphorylation on Ser955 stimulates Duox1-dependent H2O2 generation. Absence of phosphorylation on this residue in the S955A variant would decrease its sensitivity to calcium, explaining its lower basal activity. Combination of S955A and S1217A leads to a Duox1 phospho-null mutant with WT-like constitutive activity but without response to PKA stimulation. However, we cannot exclude the possibility that these mutations could also be associated with conformational changes, inactivating the enzyme as suggested by low cell surface expression of the S955A and S955A/S1217A proteins.

Duox2/DuoxA2 co-transfected cells treated with nanomolar concentrations of PMA show increased H2O2 generation associated with PMA-mediated Duox2 phosphorylations. Duox2 enzyme is 1,000 times more sensitive to PMA than Duox1, suggesting that in physiological conditions, the resting levels of diacylglycerol combined with basal calcium concentration would be sufficient to stimulate Duox2 but not Duox1. Therefore, Duox2 would be the predominant enzyme in the thyroid conferring constitutive activity to the system. A survey of the Duox2 primary sequence reveals 11 (S/T)-X-(R/K) motifs as potential target sites for PKC. Among them, six are not conserved in Duox1 and constitute good candidates for PKC-mediated phosphorylations. Their implication on Duox2 activity will be investigated.
Activation Mechanisms of Duox1–2 Isoenzymes

REFERENCES


Acknowledgments—We thank Chantal Degraef, Bernadette Bouronville, and Virginie Imbault for excellent technical assistance and Dr. M. Cappello for providing the thyroid tissue.
Activation Mechanisms of Duox1–2 Isoenzymes

FEBS Lett. 233, 74–78
Suppl. Table 1. Primer sequences for mutant constructions. Mutations in Duox1-2 were introduced by directed mutagenesis using the following sense primers. Position of the mutated amino acid is given for the wild-type untagged protein and the mutated nucleotides are underlined.

Suppl. Fig. 1. Effect of tags on Duox1-2 activity. A) H$_2$O$_2$ (ng) was accumulated during 2.5h at 37°C from Cos-7 cells co-expressing DuoxA1 or DuoxA2 (500ng DNA) with Duox1 (white; 500ng), Rho-HA-Duox1 (horizontal line; 500ng), Duox2 (black; 500ng) or HA-Duox2 (vertical lines; 500ng). Cells were stimulated during the 2.5h period with 1µM ionomycin, 1µM Fsk or 5µM PMA. Each measurement was performed in duplicate (mean ± SD). B) Corresponding western showing the amount of Duox proteins expressed in Cos-7 cells transfected in the same conditions as described above.

Suppl. Fig. 2. Loss of Duox1 activity by mutations in EF-hand motifs. H$_2$O$_2$ accumulation was performed during 2.5h at 37°C in the presence of 1µM ionomycin, 1µM Fsk or 5µM PMA from cells expressing DuoxA1 with WT (black), E839Q (grey) or E875Q (white) Rho-HA-Duox1. Each sample was tested in duplicate (mean ± SD). Expression of mutated Duox1 constructs at the cell surface relative to WT is shown in the inset.

Suppl. Fig. 3. Topological model of the Duox1 protein with the substituted amino acids used to identify the PKA target sites (S955 – T1007 – S1217).

Suppl. Fig. 4. Kinetics of Duox1 phosphorylation by Fsk. Cos-7 cells were incubated with or without fetal bovine serum (FBS) and stimulated during 10, 20, 30 or 60 min with 10µM Fsk. Phosphorylation was detected using the anti-RXX(pS/pT) antibody (P-PKA). Relative PKA-mediated Duox1 phosphorylation corrected to the total amount of Duox1 immunoprecipitated is shown on the top of the figure (non stimulated condition incubated in absence of serum was considered as 100%).

Suppl. Fig. 5. Duox1 phosphorylation is increased through the cAMP pathway. Cos-7 cells co-transfected with Rho-HA-Duox1/DuoxA1 were incubated with $^{32}$P and stimulated 30 min with 10µM Fsk, 50µM 6-MB-cAMP or co-transfected with the plasmid encoding the PKA catalytic subunit (PKA). Relative Duox1 phosphorylation corrected to the total amount of Duox1 in the immunoprecipitate is represented on the top. PKA condition tested in another experiment.

Suppl. Fig. 6. Phosphorylation of Duox1 mutated in potential phosphorylation sites. Co-transfected cells expressing DuoxA1 together with WT, S955A, T1007A, S1217A or S955A/S1217A Rho-HA-Duox1 proteins were incubated with (+) or without (-) 10µM Fsk for 30 min. Duox1 phosphorylation was corrected relative to the total amount of immunoprecipitated Duox1. Phosphorylation was detected using the anti-RXX(pS/pT) antibody (P-PKA). The mutant S955A/S1217A was tested in another experiment together with the WT Duox1 protein.
# Supplemental table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duox1 mutants:</strong></td>
<td></td>
</tr>
<tr>
<td>E839Q</td>
<td>GGCAATGGGATACCTGTCCCTCCGAACAGTTCCTG</td>
</tr>
<tr>
<td>E875Q</td>
<td>AATGGCCCTGATATCCAAGGATCAGTTCACTAGG</td>
</tr>
<tr>
<td>S955A</td>
<td>CTCTGCCGGCGAGCCCTACATCAGCCAGGA</td>
</tr>
<tr>
<td>T1007A</td>
<td>TTTGCAAGAGGTAACGTCTCATCCAGCCTTCG</td>
</tr>
<tr>
<td>S1217A</td>
<td>CACTCCGCCGCGGCGCTTCCCGGGCTTCTGG</td>
</tr>
<tr>
<td><strong>Duox2 mutants:</strong></td>
<td></td>
</tr>
<tr>
<td>E843Q</td>
<td>TACCTGTCCCTCCGAACAGTTCTAGACATCCTG</td>
</tr>
<tr>
<td>E879Q</td>
<td>TTCTTCTCAGGACCAATCTCTCCACCATGATG</td>
</tr>
</tbody>
</table>
Supplemental Figure 1
Supplemental Figure 4
Supplemental Figure 5

Phosphorylation

% Basal

kDa

200
190

200
190

DA1

Basal Fsk 6MB Basal PKA

D1DA1

32p

Duox1
Supplemental Figure 6