Targeting of the Dual Oxidase 2 N-terminal Region to the Plasma Membrane*

Received for publication, May 14, 2004, and in revised form, May 18, 2004
Published, JBC Papers in Press, May 18, 2004, DOI 10.1074/jbc.M405406200

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Dual oxidase 2 (Duox2) is a cell surface glycoprotein that probably provides thyroperoxidase with the H2O2 required to catalyze thyroid hormone synthesis. No functional H2O2-generating system has yet been obtained after transfecting Duox2 into non-thyroid cell lines, because it is retained in the endoplasmic reticulum (ER). We investigated the level of maturation of various Duox2 truncated proteins in an attempt to identify the region of Duox2 responsible for its remaining in the ER. Duox2-Q686X mutant, corresponding to the N-terminal ectodomain including the first putative transmembrane domain, was expressed in different cell lines. Carbohydrate content analysis revealed that complex type-specific Golgi apparatus (GA) oligosaccharides were present on pig Duox2-Q686X, whereas human truncated Duox2 carried only high mannose-type sugar chains characteristic of the ER. Further characterization using surface biotinylation and flow cytoometry assays indicated that pig Duox2-Q686X was present at the plasma membrane, whereas human truncated Duox2-Q686X remained inside the cell. The replacement of the last 90 residues of the human Duox2-Q686X with the pig equivalent region allowed the chimerical peptide to reach the Golgi apparatus. Pig mutants containing the complete first intracellular loop with or without the second transmembrane domain accumulated in the ER. These findings show that 1) the human Duox2-Q686X region encompassing residues 596–685 prevents mutant exportation from the ER and 2) there is a pig Duox2 retention domain in the first intracellular loop. In addition, missense mutations of four cysteines (Cys-351, -370, -568, or -582) completely inhibited the emergence of pig Duox2-Q686X from the ER compartment, indicating their importance in Duox2 maturation.

During thyroid hormone synthesis, thyroperoxidase (TPO) catalyzes the iodination of tyrosyl residues on thyroglobulin and couples hormonogenic pairs of iodothyronines molecules to form iodothyronines (1). This synthesis occurs on the outside of the apical plasma membrane of thyrocytes in the presence of hydrogen peroxide, which acts as an electron acceptor (2). The H2O2-generating system associated with TPO is a calcium-dependent NADPH:O2 oxidoreductase, which has a catalytic core containing a 1548-amino acid integral membrane flavoprotein encoded by the DUOX2 gene (3–5). A biallele inactivating mutation on the DUOX2 gene was found in a patient with a total iodine organification defect; this confirmed the essential role of Duox2, probably as a supplier of H2O2 for TPO (6). The DUOX1 gene, situated near DUOX2 on chromosome 15q15, encodes a homologous 1551-amino acid protein displaying 83% sequence similarity (5). DUOX mRNAs are also detected in epithelial cells of some secretory glands and mucosal surfaces, where these enzymes could provide H2O2 for peroxidase-mediated antimicrobial defense processes (7, 8).

The Duox glycoprotein sequence can be divided into three parts, each extending over about one-third of the molecule (Fig. 1). The C-terminal region, comprising the last six TMDs of Duox, displays high sequence similarities with the other members of the NAD(P)H oxidase family (9), indicating that it probably catalyzes the transfer of electrons across the membrane from NADPH to molecular oxygen. The large intracellular loop located in the middle part of Duox, between the first two TMDs, contains two potential calcium EF-hand binding motifs and is probably involved in the control by calcium of the conformation and activity of NADPH oxidase (10, 11). The ~600-residue-long extracellular N-terminal part of the protein is in part homologous to animal heme peroxidases and distinguishes the Duox family from other NAD(P)H oxidases. Edens et al. (12) reported that the peroxidase-like domain of Duox1, which is expressed in bacteria, catalyzes protein cross-linking by forming dityrosine bonds in the bacterial lysate. However, the role of the homologous domain of Duox2 in thyroid hormone biosynthesis is still poorly understood, and its peroxidase activity apparently duplicates that of TPO. The sequence of porcine Duox2 includes six putative N-glycosylated sites located in the N-terminal ectodomain, whereas only five such sites have been identified in the human protein. Western blots of membrane proteins from human and pig thyrocytes displayed Duox2 as two bands corresponding to two differently N-glycosylated states of Duox2 with oligosaccharide motifs accounting for 15 and 25 kDa of their molecular mass, respectively (13, 14). Only the more highly glycosylated form was resistant to endoglycosidase H treatment, indicating that it passes through the GA and also suggesting that it constitutes the mature form of the NAD(P)H oxidase at the plasma membrane (14).

No reconstitution of a functional H2O2-generating system based on Duox1 or Duox2 proteins has yet been achieved.
Human Duox expressed in CHO and COS cells remains exclusively intracellular, with no post-ER maturation (14). Some unidentified components are probably required to target the Duox proteins toward the GA or to retain Duox in the ER (6, 14). To explore this latter possibility, we expressed C-terminal-deleted mutants of human and porcine Duox2 in Hek293, CHO, and PCCL3 cells. The first mutants, termed human and pig Duox2-Q686X, respectively, included the peroxidase-like ectodomain and the first TMD (Figs. 1 and 2). This exactly matched the truncated protein probably expressed in the thyroid of a patient with the C2050T monoallelic mutation on the DUOX2 gene and exhibiting a partial iodide-organification defect phenotype (6). The other mutants used were the longer porcine Duox2-H1038X and Duox2-V1075X proteins, truncated before and after the second TMD, respectively (Fig. 1). In this study, we investigated the maturation of these mutants in terms of their N-glycosylation states and subcellular localization. The role of the cysteine residues located in the ectodomain was also investigated in the intracellular trafficking using site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Generation of Duox2 Constructs**—Human and pig DUOX2 open reading frame cDNAs were amplified by RT-PCR using 1 μg of human or pig thyroid mRNA with primers designed according to the GenBank™ database (NM_014080 and AF547237 for human and pig DUOX2 cDNA, respectively). Human and pig DUOX2 products (4.6 kb) were cloned into pcDNA3.1D/TOPO vectors (Invitrogen) and sequenced. Human and pig Duox2-Q686X were amplified by PCR using these plasmids. The sense PCR primers used for human and pig Duox2 constructs included the ATG start codon followed by 27 nucleotides. The antisense PCR primers for human and pig Duox2-Q686X were 5′-CTAGACCA-CACGGGACACGTGAGGTCCCT-3′ and 5′-CTAGATGTGACGAGCAGTCCAG-3′, respectively. The PCR products were cloned into pcDNA3.1D/TOPO vectors (Invitrogen) and sequenced. To generate stable cell lines using the Fp-Inf™ system (Invitrogen), human and pig Duox2-Q686X inserts were subcloned into pcDNA5/FRT, using KpnI/NotI.

Human and pig Duox2-(1–685) fused in-frame with green fluorescent protein (GFP) were produced by PCR using human and pig pcDNA3.1D/Duox2 plasmids as templates. The antisense PCR primers for human and pig Duox2-(1–685) were 5′-CGACCAACCGAGGACGACATGGAGATG-3′ and 5′-CTAGGTGACGAGCAGTCCAG-3′, respectively. The PCR products were cloned into pcDNA3.1D/CT-GFP-TOPO vector (Invitrogen) and sequenced. Pig Duox2-H1038X and pig Duox2-V1075X fragments were amplified by PCR using pig pcDNA3.1D/Duox2 plasmid with the antisense primer 5′-TCACACCGGTGTAATTCCTCA-CACGGGCTGTGA-3′ and 5′-CTCAAAAGGGGGCTCTGCAATGGCCAGGG-3′, respectively. The fragments were cloned into pcDNA3.1D/TOPO vector and sequenced.

The chimical construct coding the first 595 amino acids of the human Duox2 followed by amino acids 596–685 of the pig Duox2 was obtained by overlapping PCR using human and pig pcDNA3.1D/Duox2 plasmids as templates (15). The sense and antisense primers used to amplify the human Duox2-(1–596) sequence were 5′-CCCAAGCTTAT-CTCGCCGCGGAGGACAGT-CGATGGTACGGAGCACTGAGAGATGCCT-3′ and 5′-TACGATGTTGATGCGCCAAAAGGGAGTCTGGTGCTCTTCAAAAGGTCAGAAGATG-3′ (primer 2), respectively. The sense and antisense primers used to amplify the pig Duox2-(596–685) sequence were 5′-CCGCTGAGTCAAGTCCAGGAGGACAGTCTGGTGCTCTTCAAAAGGTCAGAAGATG-3′ and 5′-TACGATGTTGATGCGCCAAAAGGGAGTCTGGTGCTCTTCAAAAGGTCAGAAGATG-3′ (primer 3), respectively. The PCR products (1.8 and 0.2 kb, respectively) were purified, mixed together, and submitted to a 15-cycle PCR without adding primers and then to a 30-cycle PCR with primers 1 and 3. The PCR fusion product (2.0 kb) was then digested by HindIII/Xhol, cloned into pcDNAs/FRT plasmid and sequenced. Cysteines in pig Duox2-Q686X were replaced by glycines by site-directed mutagenesis using the QuikChange Kit (Stratagene). Primers containing the desired mutation and the PCR conditions were selected according to the manufacturer’s guidelines.

**Cell Cultures, Transfection, and Preparation of the Particulate Fraction**—Rat thyroid cells (PCCL3), developed as described previously (16), were a generous gift from Dr. R. Di Lauro (Stazione Zoologica Anton Dohrn, Napoli, Italy). They were cultured in Coon’s modified Ham’s F12 medium (VWR) supplemented with 10% fetal calf serum, 10 micromolar of bovine thyrotrpin (Sigma), 1 μg/ml insulin, 5 μg/ml transferrin, and 10 nM hydrocortisone. Fp-Inf™ CHO and Fp-Inf™ Hek293 cell lines (Invitrogen) were grown at 37 °C in Ham’s F12 medium (Invitrogen) and in Dulbecco’s modified Eagle’s medium (Invitrogen), respectively, supplemented with 10% fetal calf serum, antibiotics, and Zeocin™. When they reached 50–60% confluence, the cells were transiently transfected by using the FuGENE (Roche Applied Sciences) transfection reagent. After 24 h, 20 μM hemin and 10 mM sodium butyrate were added to the medium; the PCCL3 cells were also supplemented with 10 μg/ml forskolin. After 48 h, cells were washed with PBS and scraped into the same solution supplemented with a mixture of protease inhibitors (5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 157 μg/ml benzamidine). After centrifuging at 200 × g for 10 min at 4 °C, the cell pellet was homogenized using a motor-driven Teflon pestle homogenizer in 2 ml of 50 mM sodium phosphate buffer containing 0.25 M sucrose, 0.1 M dithiothreitol, 1 mM EDTA (pH 7.4), 0.1% SDS, before adding 50 units/ml N-glycosidase F (Roche Applied Sciences) or in 100 mM sodium citrate buffer, (pH 5.5) containing the antiprotease mixture, 2.5 mM EDTA, 0.1% SDS, before adding 50 units/ml N-glycosidase F (Roche Applied Sciences) or in 100 mM sodium citrate buffer, (pH 7.2) containing the mixture of protease inhibitors. After centrifuging at 3,000 × g for 30 min, the pellet was resuspended in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 0.25 M sucrose, 1 mM MgCl2, and the mixture of protease inhibitors.

**Deglycosylation Experiments**—Deglycosylation was performed as follows: 100 μg of particulate fraction was treated either in 50 mM sodium phosphate buffer containing 0.25 M sucrose, 0.1 M dithiothreitol, 1 mM EDTA (pH 7.4), or in 100 mM sodium citrate buffer, before adding 50 units/ml N-glycosidase F (Roche Applied Sciences) or in 100 mM sodium citrate buffer, (pH 5.5) containing the antiprotease mixture, 2.5 mM EDTA, 0.1% SDS, before adding 1 unit/ml Endoglycosidase H (Roche Applied Sciences). After a 1-h incubation, an additional 50 units/ml N-glycosidase F or 1 unit/ml Endoglycosidase H was added, and the incubation was continued for a further 1 h. Controls were performed under the same conditions except that deglycosylation enzymes were omitted. The reaction was stopped by adding concentrated Laemmli sample buffer before performing electrophoresis.

**Western Blot Analysis**—SDS-PAGE and immunoblot analyses were performed as described previously (17). The Duox2 protein was detected...
using the polyclonal IgG antipeptide raised against the 14-amino acid peptide encompassing the Leu-410-Thr-423 portion of human and porcine Duox2. This antipeptide does not detect rat Duox2 protein.

**Cell Surface Biotinylation**

Flp-In™ Hek293 and Flp-In™ CHO cells were stably transfected with pig and human pcDNA5/FRT-Duox2-Q686X, respectively, and clones were selected in a hygromycin-B-supplemented culture medium. Resistant clones were screened for positive Duox2-Q686X expression by Western blot analysis. Cell monolayers grown in 35-mm Petri dishes were washed twice with ice-cold PBS (pH 8.0) supplemented with 1 mM CaCl2 and 1 mM MgCl2 (PBS-CM) before sulfo-NHS-biotin (Pierce) was added at the concentration of 0.5 mg/ml in PBS-CM for 10 min on ice. Control cells were treated without sulfo-NHS-biotin. The reagent was removed, and the same operation was repeated once. The medium was then removed, and the remaining reactive sulfo-NHS-biotin was blocked by adding 50 mM NH4Cl in PBS-CM. The cells were then washed with PBS-CM before adding 600 l of solubilization buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM CHAPS, and the antiprotease mixture. Cells were incubated for 30 min on ice while being gently shaken and then centrifuged for 30 min at 200,000 g. The supernatant was then incubated for 2 h with 200 μl of avidin-agarose (Sigma). The biotinylated surface Duox2-Q686X and the intracellular Duox2-Q686X were separated by centrifuging (1 min, 9,000 g). The beads were washed seven times at 4 °C with extraction buffer and twice with PBS-CM, resuspended in 100 l of Laemmli sample buffer, and boiled for 5 min. 50 μl of the supernatant corresponding to the cell surface fraction and 5 μl of the supernatant corresponding to the intracellular fraction were analyzed by SDS-PAGE and immunoblot.

**Fluorescence-activated Cell Sorter Analysis**

Flp-In™ Hek293 and Flp-In™ CHO cells stably expressing pig and human Duox2-Q686X, respectively, were washed with PBS and detached from the plates using PBS containing 5 mM EDTA and 5 mM EGTA for 10 min at 37 °C before being transferred into polypropylene tubes (10⁷ cells/tube). Cells were washed three times with PBS and centrifuged at 200 g for 5 min at room temperature; the supernatant was then removed by inversion. Cells were then incubated at 4 °C with 2 ml of complete culture medium. After 1 h, they were washed with PBS and centrifuged at 400 g for 3 min. They were then incubated for 45 min at 4 °C in the dark with 400 l of complete culture medium containing 8 μl of antipeptide. Cells were washed three times with PBS and centrifuged at 400 × g for 3 min. They were then incubated for 45 min at 4 °C with 400 μl of complete culture medium containing 8 μl of antipeptide. Cells were washed three times with PBS and centrifuged as above. They were then incubated for 30 min on ice while being gently shaken and then centrifuged for 30 min at 200,000 × g. The supernatant was incubated for 2 h with 200 μl of avidin-agarose (Sigma). The biotinylated surface Duox2-Q686X and the intracellular Duox2-Q686X were separated by centrifuging (1 min, 9,000 × g). The beads were washed seven times at 4 °C with extraction buffer and twice with PBS-CM, resuspended in 100 μl of Laemmli sample buffer, and boiled for 5 min. 50 μl of the supernatant corresponding to the cell surface fraction and 5 μl of the supernatant corresponding to the intracellular fraction were analyzed by SDS-PAGE and immunoblot.

**Fig. 2.** Protein sequence alignment of human and pig Duox2-Q686X. The signal sequence includes the first 20 residues (*italic*). Among the cysteines (*black*), 7 are conserved in both species. Common amino acids and antipeptide are labeled with a *gray* and *black* background, respectively. The putative TMD and N-glycosylation motifs are surrounded by a *narrow* and *broad black border*, respectively.
Confocal Microscopy—Flp-In™ Hek293 and Flp In™ CHO cells transiently transfected with human and pig pcDNA3.1Duox2-(1–685)-CT-GFP were grown on a coverslip (Superior) and fixed with 4% paraformaldehyde at room temperature during 10 min. Cells were then washed twice for 5 min with PBS at room temperature and mounted in Mowiol. Cells were viewed with a ZEISS-LSM 510 laser scanning confocal microscope using a ×63/1.4 NA oil immersion objective lens. GFP was excited at 488 nm, and fluorescence emission was collected through a 515/545-nm bandpass filter. The pinhole was set at one Airy unit.

RESULTS

Expression and Deglycosylation Studies of Human and Pig Duox2-Q686X in Hek293, CHO, and PCCL3 Cell Lines—Two non-thyroid cell lines (Hek293 and CHO) and a rat thyroid cell line (PCCL3) were transiently transfected with expression vectors encoding the human or pig Duox2-Q686X mutant. During the transfection period, the medium was supplemented with hemin because heme incorporation might affect flavoprotein maturation processing as previously reported for TPO and Noxx2 (18–20), and PCCL3 were stimulated with forskolin to express the differentiation markers. Membrane proteins were then analyzed by Western blot, and human truncated Duox2 was visualized as a single immunoreactive band with an apparent molecular mass of 85 kDa (Fig. 3, lanes H). Surprisingly, the polyclonal antipeptide immunodetected the pig Duox2-Q686X protein as a doublet (Fig. 3, lanes F). One band had the same apparent molecular mass of 85 kDa as the human mutant, but a second protein was revealed as a broad band of ~100 kDa. Each band could correspond to a specific glycosylation state of the protein, as previously observed for the full-length Duox2 (13). Indeed, the calculated molecular mass of Duox2-Q686X mutants is only 70 kDa, but they contain all the putative N-glycosylation sites of human and porcine Duox2, numbering five and six sites, respectively. Deglycosylation experiments were therefore performed to check this assumption. The particulate fractions were digested with N-glycosidase F (NGaseF) or endoglycosidase H (EndoH) and analyzed by Western blot. The treatment with NGaseF or EndoH of Hek293, CHO, and PCCL3 particulate fractions expressing human Duox2-Q686X shifted the 85-kDa form to a 70-kDa protein with the predicted molecular mass of the non-glycosylated protein (Fig. 4, A–C). The NGaseF treatment of protein extracts from Hek293, CHO, and PCCL3 cells expressing the pig Duox2-Q686X mutant converted the doublet into a single band, reducing the apparent molecular mass of the 85 and 100-kDa forms by 15 and 25 kDa, respectively (Fig. 4, D–F, lanes F). However, the 100-kDa form of pig Duox2-Q686X was not affected by digestion with EndoH, whereas the 85-kDa protein was still converted into the 70-kDa form (Fig. 4, C–E, lanes H). Taken

Fig. 3. Western blot analysis of human and pig Duox2-Q686X mutants expressed in different cell lines. Flp-In™ Hek293 (A) and Flp-In™ CHO (B) cells were transiently transfected for 48 h with human (H) or pig (P) pcDNA3.1/Duox2-Q686X. The particulate fraction of the cells (35 μg) were then processed by SDS-PAGE and immunoblot as described under “Experimental Procedures.”

Fig. 4. Analysis of the glycosylation states of human and pig Duox2-Q686X mutants. Flp-In™ Hek293 (A–D), Flp-In™ CHO (E–H), and PCCL3 (C–F) cells were transiently transfected for 48 h with human (A–C) or pig (D–F) pcDNA3.1/Duox2-Q686X, and the particulate fractions of the cells were treated with or without (−) N-glycosidase F (F) or endoglycosidase H (H) as described under “Experimental Procedures.”

Fig. 5. Intracellular and cell surface distribution of human and pig Duox2-Q686X mutants. Flp-In™ Hek293 cells stably transfected with pig Duox2-Q686X (A) and Flp-In™ CHO cells stably transfected with human Duox2-Q686X (B) were incubated with (+) or without (−) the cross-linker (s-NHS-B). Intracellular (Intra) and cell surface (Surface) forms of Duox2-Q686X were separated using avidin-agarose. The samples were then analyzed by SDS-PAGE.

Fig. 6. Flow cytometric analysis of the cell surface expression of human and pig Duox2-Q686X mutants. A, histogram of fluorescence intensity (arbitrary units) showing the binding of the antipeptide to Flp-In™ Hek293 expressing the pig Duox2-Q686X mutant (open histogram) or without the Duox2 mutant (dark gray histogram). B, histogram of fluorescence intensity showing the binding of the antipeptide to Flp-In™ CHO expressing the human Duox2-Q686X mutant (white histogram) or without the Duox2 mutant (dark gray histogram).
together, these findings indicate that the glycosylation of pig truncated Duox2 protein passed through the GA, unlike the corresponding human protein, which exhibited only ER-specific oligosaccharide motifs.

**Cell Surface Expression of Duox2-Q686X Mutants**—The differences observed in the glycosylation states of the human and porcine mutants might reflect differing subcellular distributions of the proteins. In particular, the evidence for a GA-specific glycosylation of the pig protein suggested that it could be targeted to the plasma membrane. Cell surface biotinylation experiments, carried out with a non-permeable reagent, were performed on the Hek293 and CHO cell lines stably expressing pig and human Duox2-Q686X mutants, respectively. Sulfo-NHS-biotin treatment revealed that a fraction of the porcine mutant 100-kDa form was present at the cell surface, whereas the partially glycosylated protein could not be biotinylated (Fig. 5A). Fig. 5B shows the human Duox2-Q686X 85-kDa protein was not expressed at the plasma membrane. The presence of the Duox2-Q686X protein at the plasma membrane was also measured by flow cytometry analysis. Fig. 6A demonstrates the binding of the antipeptide to the plasma membrane of non-permeabilized cells expressing pig Duox2-Q686X, whereas no such fluorescence intensity shift was observed with human Duox2-Q686X (Fig. 6B). Duox2-(1–685)-CT-GFP fusion protein expressed for 48 h in both cell lines also provided evidence of a species-dependent subcellular distribution of each protein. Again, the porcine fusion protein expressed in Hek293 and CHO cells was detected in the Western blot experiment as a doublet, whereas the human tagged protein appeared as a single immunoreactive band (data not shown). When analyzed by confocal microscopy, the porcine fusion protein was found at the cell plasma membrane (Fig. 7, A and B), but we also observed intracellular fluorescence on other cellular sections. The human GFP-tagged mutant was visualized only as intracellular spots (Fig. 7, C and D).

**Expression and Deglycosylation Studies of the Human-Pig Chimerical Duox2-Q686X Protein Expressed in the Hek293 Cell Line**—Proteasome-dependent endocytosis and degradation pathway has been reported to be implicated in the turnover of receptors (21). Hek293 cells stably expressing the human Duox2-Q686X mutant were therefore treated for 24 h with a specific proteasome inhibitor, the MG132 compound, to test the involvement of the ubiquitin/proteasome system in the rapid degradation of the human mature Duox2 form. Western blot analysis revealed no novel band over the ER-specific 85-kDa form but rather an increased amount of the unglycosylated human Duox2-Q686X protein (data not shown).

A chimerical gene was constructed by fusing in-frame the human DUOX2 cDNA sequence coding the N-terminal ectodomain with the pig DUOX2 cDNA sequence coding from the first TMD to amino acid 685 (Fig. 8A). This construct was transiently expressed in Hek293 cells for 48 h. Membrane proteins were then submitted to Western blot analysis. The antipeptide antibody revealed the chimerical peptide as a doublet: one band had the same apparent molecular mass of 85 kDa as the human Duox2-Q686X mutant, but a second protein with a higher molecular mass was visualized under 100 kDa (Fig. 8B). Deglycosylation studies demonstrated that this latter form had passed through the GA because the N-linked oligosaccharides were not affected by the endoglycosidase H treatment (Fig. 8C). These findings indicate that the replacement of the last 90 amino acids of the human Duox2-Q686X mutant by the equivalent pig sequence allowed the chimerical peptide to reach the GA. Duox2-Q686X protein sequence alignment of both species showed that the human mutant contains a cysteine residue at position 671 that is not present in the pig sequence (Fig. 2). This amino acid located in the human C-terminal region could be responsible for the mutant ER retention by linking another protein through a disulfide bond. By site-directed mutagenesis, we changed cysteine 671 into a glycine using the human
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From 596 to 685.

A single band was also immunodetected in CHO cells expressing the chimerical human-pig Duox2-Q686X protein (Fig. 8D). Thus, the presence of the first intracellular loop sequence, with or without the second TMD, inhibited the GA-specific modification of carbohydrate motifs and the maturation of the Duox2 N-terminal ectodomain.

Role of Pig Duox2-Q686X Cysteines during the Maturation Process—The pig Duox2-Q686X protein contains nine cysteines, one in the signal peptide, six in the extracellular part, and two vicinal cysteines located inside the first putative TMD (Fig. 10A). Each pig Duox2-Q686X cysteine, except the one in the signal peptide, was replaced separately by a glycine. CHO cells were then transiently transfected, and particulate fractions were submitted to Western blot analysis. Replacement of the Cys-124, -265, -606, and -607 cysteines had no effect on the N-linked glycosylation process because the doublet was still immunodetected (Fig. 10B). However, the substitution of Cys-351, -370, -568, or -582 prevented the expression of the 100-kDa form. These data indicate that these four cysteine residues located in the ectodomain of Duox2 play a critical role in its maturation and emergence from the ER.

**DISCUSSION**

When the human complete Duox2 protein is expressed in non-thyroid cell lines, it fails to leave the ER and no mature Duox2 protein is visualized by Western blot (14). The hypothesis of De Deken et al. was that other thyroid-specific protein partners were needed for the Duox2 protein to progress to the GA. The mechanism by which Duox2 is retained in the ER has not yet been elucidated. ER accumulation could be because of the presence of a retention domain in the Duox2 protein. In this study, we explored this possibility by deleting the C-terminal part of the enzyme, after the first or the second TMD, to find out whether the truncated polypeptides were retained in the ER or targeted to the membrane. Human and pig Duox2-Q686X mutants, expressed in two non-thyroid cell lines and in a rat thyroid cell line, were shown to have differing N-glycosylation states. The human mutant carried only high mannose carbohydrates, indicating it did not leave the ER, whereas its porcine counterpart had acquired complex oligosaccharide motifs in the GA. Fluorescence-activated cell sorter experiments demonstrated that the N-terminal...
region of Duox2 is actually an ectodomain, in accordance with the topology predicted by the analysis of its primary sequence (3, 5). Furthermore, cell surface biotinylation experiments revealed that only the endoglycosidase H-resistant form of pig Duox2-Q686X was exposed to the extracellular medium, reinforcing the hypothesis that the mature and active NADPH oxidase located at the apical membrane of thyrocytes solely involves the highly glycosylated form of Duox (14).

Sequence analysis of the human and pig Duox2-Q686X revealed 19% heterogeneity, with an additional putative glycosylation site number and amino acid composition between the human and pig ectodomain do not differ significantly, showing that the ER retention signal is effective in controlling the ER sorting of a longer protein and possibly that of the full-length human Duox2. However, this study also showed that another ER retention domain is located downstream the residue 685.

In addition to identification of internal peptide sequences involved in the control of Duox2 targeting, we also investigated the effect on Duox2 maturation of some amino acid substitutions. Cysteine and proline residues play essential functions in the formation of the correct three-dimensional structure of a polypeptide through disulfide bonds and restriction angles, respectively. Most of the proline residues of human and pig Duox2 are present in the N-terminal ectodomain, suggesting that the folding of this region plays a critical role. It has been reported that protein trafficking disorders can result from the substitution of a single amino acid, leading to failure of protein exportation from the ER (25). Hishinula et al. (26) demonstrated that goiters are due to the retention of thyroglobulin in the ER, which is caused by specific cysteine substitutions. Site-directed mutagenesis allowed us to investigate the role of the Duox2-Q686X cysteines during maturation. Four conserved cysteines within the Pro-596-Val-685 region of human Duox2 are present in the N-terminal ectodomain, suggesting that the folding of this region plays a critical role. It has been reported that protein trafficking disorders can result from the substitution of a single amino acid, leading to failure of protein exportation from the ER (25). Hishinula et al. (26) demonstrated that goiters are due to the retention of thyroglobulin in the ER, which is caused by specific cysteine substitutions. Site-directed mutagenesis allowed us to investigate the role of the Duox2-Q686X cysteines during maturation. Four conserved cysteines within the Pro-596-Val-685 region of human Duox2 were unable to acquire GA-specific glycosylation, indicating they remained blocked in the ER, whereas adding the 239-amino acid GFP to the C-terminal part of pig Duox2-(1–685) did not inhibit its cell surface targeting. The premature stop codon did not generate a C-terminal ER retention signal, such as the KDEL (23) or the dilyssine KKKX motif (24). Consequently, an ER retention domain must be present within the first cytoplasmic loop of the pig Duox2, between residues 686 and 1037. This region contains the two putative EF-hands of Duox proteins that could possibly account for interactions with ER-localized proteins. The porcine mutant constitutes a tool that could be used to identify the minimum peptide sequence within the intracellular loop that confines the pig Duox2 mutant in the ER.

Further experiments are required to determine whether this ER retention domain is also present in the same region of the human Duox2.
cysteines in human and pig Duox2 truncated proteins (Cys-
351, -370, -568, and -582) have an essential function in the
secretion pathway. The disappearance of these specific amino acids inhibited GA carbohydrate remodeling, showing that mu-
tants were retained in the endoplasmic compartment, likely
because of their abnormal three-dimensional structure.

Finally, the pig truncated Duox2-Q686X protein and the
highly glycosylated human-pig Duox2-Q686X chimera ex-
pressed in Hek293 cells will also be useful models for exploring
the function of the peroxidase-like extracellular domain of hu-
man and pig Duox2. On the one hand, Lambeth’s group (12)
has shown that prokaryotes expressing the human Duox1 peroxi-
dase-like region catalyzed the cross-linking of free tyrosine
ethyl ester, although sequence alignment revealed that the
crucial residues for catalytic activity in animal heme peroxi-
dase, such as the distal and proximal histidines (27), are not
conserved in human Duox1/2 proteins (12). However, no data
have to date been reported about the activity of the homologous
part of Duox2. On the other hand, the Duox-specific and per-
oxidase-like N-terminal region might participate in the mech-
anism of H$_2$O$_2$ formation or release by conferring on the Duox2-
based NAD(P)H oxidases their unique ability to release H$_2$O$_2$
instead of superoxide (8, 28, 29).

Acknowledgment—We thank Dr. Sacha Lebel from Laboratoire de
Microbiologie-Immunologie-Virologie of the University of Paris XI for
assistance in plasmids sequencing.

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J. Biol. Chem. 2004, 279:30244-30251.
doi: 10.1074/jbc.M405406200 originally published online May 18, 2004