Identification of the Maturation Factor for Dual Oxidase

EVOLUTION OF AN EUKARYOTIC OPERON EQUIVALENT

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Dual oxidase 2 (DUOX2), an NADPH:O₂ oxidoreductase flavoprotein, is a component of the thyroid H₂O₂ generator crucial for hormone synthesis at the apical membrane. Mutations in DUOX2 produce congenital hypothyroidism in humans. However, no functional DUOX-based NADPH oxidase has ever been reconstituted at the plasma membrane of transfected cells. It has been proposed that DUOX retention in the endoplasmic reticulum (ER) of heterologous systems is due to the lack of an unidentified component required for functional maturation of the enzyme. By data mining of a massively parallel signature sequencing tissue expression data base, we identified an uncharacterized gene named DUOX maturation factor (DUOXA2) arranged head-to-head to and co-expressed with DUOX2. A paralog (DUOXA1) was similarly linked to DUOX1. The genomic rearrangement leading to linkage of ancient DUOX and DUOXA genes could be traced back before the divergence of eutherians. We demonstrate that co-expression of DUOXA2, an ER-resident transmembrane protein, allows ER-to-Golgi transition, maturation, and translocation to the plasma membrane of functional DUOX2 in a heterologous system. The identification of DUOXA genes has important implications for studies of the molecular mechanisms controlling DUOX expression and the molecular genetics of congenital hypothyroidism.

Generation of H₂O₂ at the apical membrane of thyroid follicular cells is essential for iodination of thyroglobulin by thyroid peroxidase and constitutes the rate-limiting step of thyroid hormone synthesis. Dual oxidases (DUOX1 and DUOX2)2 appear to constitute the catalytic core of the H₂O₂ generator (1, 2). They are large homologs of the phagocyte gp91phox/Nox2 NADPH-dependent oxidase with an N-terminal extension comprising a peroxidase-like domain. Although the crucial role of DUOX2 in thyroid hormonogenesis has been substantiated by reports of severe congenital hypothyroidism in patients with biallelic nonsense mutations (3), the understanding of structure, function, and regulation of DUOX has remained limited. The major obstacle for molecular studies of DUOX is the lack of a suitable heterologous cell system for DUOX-based functional NADPH oxidase expression. Transfected cells completely retain DUOX in the endoplasmic reticulum (ER) (4–8), suggesting that an unidentified component, essential for DUOX maturation, may be specifically expressed in tissues containing the functional enzyme.

EXPERIMENTAL PROCEDURES

Data Mining and Computational Analysis—Massively parallel signature sequencing (MPSS) data (9) were obtained from the NCBI Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/; records GSE1747 and GPL1443). A thyroid specificity score, as defined by longeneel et al. (9), was calculated for signatures with frequency >100 tags per million (~30 mRNA copies/cell) in the thyroid/parathyroid library. Tags with scores >1 were mapped to the human genome assembly using BLAST. DUOXA homologs were identified by tBLASTn searches against the NCBI nr database and trace archive and BLAT queries (at genome.ucsc.edu/) against assembled whole genome sequences. Orthologs were operationally defined as reciprocal best BLAST hits. Gene structures were deduced by spliced alignment maintaining maximum homolog similarity of the open reading frames (ORFs) and consensus splice junctions. Cladograms were constructed from ClustalX alignments (BLOSUM weight matrix, excluding gaps) using the Jones, Taylor, and Thornton (ITT) substitution model in PHYML 2.4.4 (10). SignAlp 3.0 (11) and Phobius (12) were used to analyze signal peptides, transmembrane helices, and topology.

Northern Blot Analysis—A human multiple tissue Northern blot (Origen) was hybridized with DUOX2A (125–470 of DQ489734) and DUOX1A (1244–1623 of BC020841) probes.

Heterologous Expression of DUOX2 and DUOXA2 Constructs—cDNA was synthesized with Superscript reverse transcriptase (Invitrogen) by oligo(dT) priming of total RNA from a normal human thyroid gland. The DUOX2 and DUOXA2 ORFs were amplified using native Pfu polymerase (Stratagene) and cloned into pcDNA3.1 (Invitrogen). Epitope-tagged constructs and fusions with enhanced green fluorescent protein (EGFP) were prepared by replacement or splicing-by-overlap extension using specifically designed primers. All constructs were verified by sequencing. HeLa cells were cultured and transfected as described (13). Confocal Laser Scanning Microscopy—Indirect immunofluorescence of permeabilized cells has been described previously (13). For surface staining, cells were incubated with anti-HA clone 3F10 and/or mouse anti-c-myc. For intracellular staining, cells were incubated with mouse anti-HA clone 12CA5 and/or mouse anti-c-myc clone 9E10 (both from Roche Applied Science) at 1 μg/ml in Hank’s buffered saline solution/10 mM Hepes, pH 7.4, 1% bovine serum albumin at 4 °C. Rabbit anti-calnexin was obtained from StressGen. Images were captured on a Nikon Eclipse E800 equipped with PCM2000.

Analysis of N-Glycosylation—Postnuclear supernatants (in 50 mM Tris/ HCl, pH 8.0, 150 mM NaCl, and protease inhibitors) were adjusted to 0.5% SDS, 0.4 mM dithiotreitol and denatured, at room temperature, for 30 min. Samples were deglycosylated with N-glycosidase F (PNGase F) and endoglycosidase H (Endo H) (both from New England Biolabs) according to manufacturer’s recommendations, followed by SDS-PAGE under reducing conditions and Western blotting as described (13).

Measurement of H₂O₂ Generation—Release of H₂O₂ was determined by reaction with cell-impermeable 10-acetyl-3,7-dihydroxyphenoxazine (14) (Amplex Red reagent, Invitrogen) in the presence of excess peroxidase, producing fluorescent resorufin. Briefly, cell monolayers were incubated, with or without 10 μM diphenylethionium (DPI), in Dulbecco’s phosphate-buffered saline supplemented with 50 μM Amplex Red reagent and 0.1 unit/ml horse-radish peroxidase for 1 h at 37 °C. Relative fluorescence units (excitation/ emission: 535/595) were corrected for Amplex Red oxidation in wells containing non-transfected cells and converted into H₂O₂ concentrations using a calibration curve. Renilla luciferase activity from co-transfected pRL-Tk plasmid (Promega) was used as internal control as described (13).

RESULTS AND DISCUSSION

Identification of Novel Genes in the DUOX1/DUOX2 Intergenic Region—We used MPSS data for 32 normal human tissues (9) to identify novel transcripts with predominant expression in thyroid gland. One of the extracted tags mapped to an uncharacterized locus (LOC405753) oriented head-to-head to DUOX2 in the ~16-kbp DUOX1/DUOX2 intergenic region. For reasons outlined below, we called the corresponding gene DUOX maturation factor 2 (DUOXA2).3 Based on human-mouse homology (Riken clone 9030623N16Rik), and supported by contig assembly of expressed sequence tags (ESTs), it comprises six

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1S–3S and Table 4S.

2 The abbreviations used are: DUOX, dual (domain) oxidase; BLAST, basic local alignment search tool; DPI, diphenyleneiodonium; EGFP, enhanced green fluorescent protein; ER, endoplasmatic reticulum; EST, expressed sequence tag; HA, hemagglutinin; MPSS, massively parallel signature sequencing; ORF, open reading frame; PNGase F, N-glycosidase F; Endo H, endoglycosidase H; contig, group of overlapping clones.

3 The gene name and symbol have been approved by the HUGO Gene Nomenclature Committee.

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exons, confirmed by reverse transcription-PCR amplification from human thyroid tissue (GenBank™ accession number DQ489734). The putative transcriptional start site of the DUOXA2 transcript is located in a GC-rich region upstream of the genomic region (Fig. 1A). This site is 135 bp from the 5′-terminus of a spliced DUOX2 EST (BI045475) on the opposite strand. A single polyadenylation signal (Fig. 1A) is supported by all mapped 3′-ESTs. We confirmed a specific transcript of the expected size (1.3 kbp) by Northern blot analysis (Fig. 1B), which also validated the expression profile of DUOX2 mRNA was by far the most abundant in thyroid, with lower levels in salivary glands reflecting the known expression profile of DUOX2 (1, 2, 15).

The DUOXA2 ORF is initiated within a Kozak consensus (gccgATG) and spans all six exons. The encoded 320-amino acid protein was strongly predicted to comprise five membrane-integral regions, including a reverse signal anchor with external N terminus (type III) (Fig. 1C). The three N-glycosylation consensus sequences for N-glycosylation are clustered within an extended external loop connecting the second and third transmembrane helices.

We identified a single DUOXA2 paralog in the human genome. We will refer to this locus, annotated as “homolog of Drosophila Numb-interacting protein,” as DUOXA1. It is immediately adjacent, in tail-to-tail orientation to DUOXA2 and extends, via untranslated exons, into the DUOX1 promoter region. DUOXA1 mRNA was predominantly expressed in thyroid and, at lower level, in esophagus (Fig. 1B). Two transcripts of ~2.9 and ~3.5 kbp were detected, compatible with alternative splicing of 5′-untranslated exons and the use of alternative 3′-polyadenylation signals (data not shown). The DUOXA1
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Characterization of DUOX2 as ER-resident Protein—DUOX2 could be an integral part of a DUOX2 complex, endowing a holocomplex with the ability to exit the ER and reach the plasma membrane. We, therefore, determined whether myc-tagged DUOX2 alone or in combination with DUOX2 would be detectable at the plasma membrane. Of several constructs tested, only DUOX2 with N- (myc-DUOX2) or C-terminal (DUOX2-myc/His) attached myc tags were fully functional in rescuing DUOX2 activity as assessed by H2O2 generation and HA-DUOX2 membrane targeting (data not shown). However, neither myc-DUOX2 nor DUOX2-myc/His was detectable at the plasma membrane (data not shown), although they had the expected size on Western blot analysis (Fig. 3A) and intracellularly co-localized with HA-DUOX2 (Fig. 3B).

To exclude that this was due to a discrepancy between the modeled and actual DUOX2 membrane topology or due to masking of the N-terminal epitope tag, we fused EGF/myc to the C terminus of DUOX2 (an N-terminal fusion was not functional). As shown in Fig. 3C, DUOX2-EGF/myc did not co-localize with HA-DUOX2 at the plasma membrane, the latter delineated by anti-HA surface staining. The intracellular distribution of DUOX2-EGF/myc (and of DUOX2-myc/His) showed a similar distribution pattern as the ER-marker calnexin (Fig. 3D).

To further corroborate that DUOX2 is indeed an ER-resident protein, we analyzed the maturation of DUOX2-myc/His N-glycosylation in cells co-expressing HA-DUOX2. We found that the N-glycans of DUOX2-myc/His were exclusively of the high-mannose type (Fig. 3E). In contrast, detection of DUOX2 in the same samples demonstrated, again, that about half of DUOX2 protein had been subject to Golgi modification of its glycosylation (data not shown, compare Fig. 2C). Collectively, these results indicate that DUOX2 is not an integral part of a DUOX2 enzyme complex at the plasma membrane but an ER-resident protein promoting ER exit and maturation of DUOX2. It should be noted that N-glycosylation of DUOX2 supports our topology model (Fig. 1C), since the apparent molecular weight of the N-glycan moieties (~10 kDa) indicates N-glycosylation of all three consensus sites.
two protostomal DUOXA-homologs analyzed were not genetically linked to duox; in fruit flies, there is also evidence for a distinct functional speciation. The Drosophila DUOXA-homolog (mol-PA) encodes a plasma membrane protein (mol-PA) implicated in the polarized recruitment of a cytosolic signal mediator (Numb) to the plasma membrane (18). That mol-PA may not cooperate with Drosophila Duox is also suggested by the distinct phenotypes caused by deficiency of mol (memory loss; listed as CG4482 in Ref 19) or Drosophila duox (defective gut immunity) (20). Remarkably, this functional divergence relates to a distinct topology prediction of the region likely crucial for functional speciation of mol-PA and DUOXA. In DUOXA, the second and third transmembrane helices are connected by an extended luminal loop consistent with N-glycosylation of DUOX2 (Figs. 1C and 3E). In mol-PA, the equivalent region harbors a binding motif crucial for recruitment of Numb (18), which, therefore, would have to be cytosolic. Analysis of the residue-wise posterior probabilities for a given state (inside/outside/transmembrane) in the 1-best Phobius topology models indeed supports such a scenario of distinct membrane topologies: the profile of transmembrane probabilities in mol-PA, but not vertebrate DUOXA, indicates the potential for two additional membrane-spanning helices (Fig. 1D), which would flank the Numb-binding motif resulting in its cytosolic exposure.

If, as we propose, DUOX expression in Drosophila does not require mol, what could have been the advantage of a DUOX/DUOXA system that caused its maintenance in deuterostomes over more than 500 million years (21) of divergent evolution? For unexplained reasons, in sea urchin eggs (22) and likewise in follicular thyroid cells (4), the bulk of DUOX protein is not detected at the cell surface but in intracellular compartments, which could provide a stimulus-recruitable pool. Thus, from an evolutionary perspective, the emergence of DUOXA may have provided an additional level of DUOX regulation, specifically, the control of DUOX translocation to the plasma membrane. The ability to reconstitute active DUOX enzyme will provide the tool to investigate the molecular mechanisms underlying DUOX expression in diverse model systems.

REFERENCES

1. Dupuy, C., Ohayon, R., Valent, A., Noel-Hudson, M. S., Deme, D., and Virion, A. (1999) J. Biol. Chem. 274, 37265–37269.
2. de Deken, X., Wang, D., Many, M. C., Costagliola, S., Libert, F., Vassart, G., Dumont, J. E., and Miot, F. (2000) J. Biol. Chem. 275, 23227–23233.
3. Moreno, J. C., Bikkir, H., Kempers, M. J., van Trotsenburg, A. S., Baas, F., de Vijlder, J. J., Vulsma, T., and Ris-Stalpers, C. (2002) N. Engl. J. Med. 347, 95–102.
4. de Deken, X., Wang, D., Dumont, J. E., and Miot, F. (2002) Exp. Cell Res. 273, 187–196.
5. Morand, S., Charras, M., Kaniewski, J., Deme, D., Ohayon, R., Noel-Hudson, M. S., Virion, A., and Dumuy, C. (2003) Endocrinology 144, 1241–1248.
6. Morand, S., Aghandjii, D., Noel-Hudson, M. S., Nicolas, V., Buisson, S., Macon-Le Maitre, L., Gudelous, S., Kaniewski, J., Ohayon, R., Virion, A., and Dumuy, C. (2004) J. Biol. Chem. 279, 30284–30291.
7. Wang, D., de Deken, X., Milenkovic, M., Song, Y., Pirson, I., Dumont, J. E., and Miot, F. (2005) J. Biol. Chem. 280, 3096–3103.
8. Ameziane El-Hassani, R., Morand, S., Boucher, J. L., Frapart, Y. M., Apostolou, D., Aghtandji, D., Guethieux, S., Ohayon, R., Noel-Hudson, M. S., Lucas, L., Lalaouin, K., Virion, A., and Dumuy, C. (2005) J. Biol. Chem. 280, 30046–30054.
9. Jongereel, C. V., Delorenzi, M., Iseli, C., Zhou, D., Hauenschlaedt, C. D., Krebetkova, J., Kuznetsova, D., Stevenson, J. B., Straussberg, R. L., Simpson, A. J., and Vasicek, T. J. (2005) Genome Res 15, 1087–1094.
10. Guindon, S., and Gascuel, O. (2003) Syst. Biol. 52, 696–704.
11. Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) J. Mol. Biol. 340, 783–795.
12. Kall, L., Krogh, A., and Sonnhammer, E. L. (2004) J. Mol. Biol. 338, 1027–1036.
13. Grasberger, H., Ringkanontun, U., Brahmanic, P., Abramowicz, M., Vassart, G., and Rieffet, S. (2005) Mol. Endocrinol. 19, 1779–1791.
14. Zhou, M., Dovz, Z., Panzuh-Koloshina, N., and Haugland, R. P. (1997) Anal. Biochem. 253, 162–168.
15. Gettier, M., Witta, J., Baillie, J., Lorkom, E., and Leto, T. L. (2003) FASEB J. 17, 1502–1504.
16. El Hassani, R. A., Benares, N., Caillo, T., Balbot, M., Sabourin, J. C., Belotte, V., Morand, S., Guethieux, S., Aghtandji, D., Ohayon, R., Kaniewski, J., Noel-Hudson, M. S., Illart, J. M., Schlabumager, M., Virion, A., and Dumuy, C. (2005) J. Physiol. 593–942.
17. Gavalas, A., Dixon, J. E., Brayton, K. A., and Zalkin, H. (1993) Mol. Cell. Biol. 13, 4784–4792.
18. Qin, H., Percival-Smith, A., Li, C., Jia, C. Y., Gloor, G., and Li, S. S. (2004) J. Biol. Chem. 279, 11034–11032.
19. Dubnaou, J., Chiang, A. S. G., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Baldoc, F., Scott, R., Cota, U., Broger, C., and Tully, T. (2003) Curr. Biol. 13, 286–296.
20. Ha, E. M., Oh, C. T., Bae, Y. S., and Lee, W. J. (2005) Science 310, 867–850.
21. Dousery, E. J., Snell, E. A., Baptiste, E., Deloux, F., and Philippe, H. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15386–15391.
22. Wong, J. L., Creton, R., and Wessel, G. M. (2004) Dev. Cell 7, 801–814.
23. Pachucki, J., Wang, D., Christophe, D., and Miot, F. (2004) Mol. Cell. Endocrinol. 214, 53–62.

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**FIGURE 3.** Characterization of DUOX2 as ER-resident protein. A, Western blot analysis of myc-tagged DUOX2 constructs. B, confocal microscopy reveals intracellular co-localization of DUOX2-myc/His with HA-DUOX2 in permeabilized cells. C, DUOX2-EGFP/myc allows functional rescue of HA-DUOX2 (visualized by surface staining) but does not co-localize with HA-DUOX2 at the plasma membrane. D, intracellular distribution of myc-tagged DUOXA2 constructs. E, DUOX2-myc/His and DUOX2-EGFP/myc exhibit distinct N-glycan moieties that are not subject to Golgi modification in cells co-expressing HA-DUOX2. Scale bars in B–D represent 10 μm.

An Evolutionary Perspective on the Physiological Role of DUOXA—The DUOX/DUOXA transcriptional unit is an excellent example of bidirectional transcription of tightly linked genes that are not structurally related but are involved in the same pathway; an arrangement considered equivalent to a prokaryotic operon (17). In contrast to the organization in deuterostomes, the

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