Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox

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Introduction

Reactive oxygen generation is well characterized in phagocytes where the respiratory burst oxidase (termed nicotinamide adenine dinucleotide phosphate [NADPH]*-oxidase) catalyzes NADPH-dependent reduction of molecular oxygen to generate superoxide and secondarily metabolites including hydrogen peroxide (Babior, 1995). In the phagocyte, reactive oxygen generation is robust, and high levels of reactive oxygen species participate in bacterial killing. In recent years, it has become clear that nonphagocytic cells also generate reactive oxygen, albeit at lower levels (Cross and Jones, 1991). The origin and function of this reactive oxygen has not been clear and has been attributed variously to "leaky" mitochondrial respiration, the activity of enzymes such as xanthine oxidase, or to the phagocyte NADPH-oxidase itself. In some cases, the reactive oxygen-generating system has properties reminiscent of the phagocyte oxidase (Emmendorfer et al., 1993; Griendling et al., 1994), but it has often been difficult to demonstrate significant expression of critical phagocyte oxidase components in these cell types (Emmendorfer et al., 1993), suggesting the existence of one or more homologues of the phagocyte NADPH-oxidase.

The phagocyte NADPH-oxidase consists of multiple subunits including gp91phox, the catalytic moiety (Babior, 1995; Yu et al., 1998). In activated cells, this subunit is associated with the plasma membrane (or with the phagosomal membrane, which is derived from the plasma membrane).
and consists of a COOH-terminal flavoprotein domain containing the NADPH-binding site (Rotrosen et al., 1992; Segal et al., 1992; Taylor et al., 1993; Takeshige and Sumimoto, 1994) and an NH2-terminal hydrophobic region comprised of five to six transmembrane α helices and harboring the two heme groups (Cross et al., 1995; Nisimoto et al., 1994; Yu et al., 1998). The structure of the enzyme permits the coupling of the oxidation of intracellular NADPH to the reduction of molecular oxygen to generate extracellular or phagosomal superoxide. Myeloperoxidase (MPO) is secreted extracellularly or into the phagosome, permitting reactive oxygen generated by the phagocyte oxidase to support hypochlorous acid generation in the extracellular/phagosomal compartment (Hampton et al., 1998; Nauseef, 1998).

Based on the hypothesis that reactive oxygen generation in nonphagocytic cells originates in part from homologues of the phagocyte oxidase, we have searched for and molecularly cloned homologues of gp91phox. The first of these, Nox1 (also termed Mox1, NOH-1), is expressed in nonphagocytic cells, including colon epithelia and vascular smooth muscle (Suh et al., 1999), and functions in regulating cell growth and cell transformation. Alternative splicing of Nox1 to generate a portion of the membrane domain produces a proton channel (Banfi et al., 2000) with properties similar to voltage-gated channels. Additional homologues of gp91phox that are similar in size to gp91phox (~65 kD) have been reported recently (Lambeth et al., 2000; Cheng et al., 2001).

In the present study, we describe large molecular weight homologues of gp91phox termed Duox, which are present in human and Caenorhabditis elegans. The term Duox, referring to dual oxidase, has been accepted by the Human Genome Organisation International Committee on Gene Nomenclature. A partial sequence of Duox2 was reported recently and termed p138Tox; the latter sequence did not contain the peroxidase homology domain (Dupuy et al., 1999). h-Duox1 and h-Duox2 were 77% identical at the amino acid level. These sequences were independently cloned recently and reported by De Deken et al. (2000).

Duox homologues in C. elegans

A BLAST search of the C. elegans genomic database using as a query the protein sequence of gp91phox identified two homologous genes contained in cosmid F56C11 and F53G12. The Ce-Duox1 conceptual transcript (sequence data available from GenBank/EMBL/DDBJ under accession no. AF267981) is predicted to encode a protein of 1,521 amino acids (177 kD), nearly three times the size of gp91phox (Fig. 1). A consensus Kozak sequence, ATG, was present at the translation start codon. The cDNA for h-Duox2 showed a 4,647 base pair ORF (sequence data available from GenBank/EMBL/DDBJ under accession no. AF229855) that is predicted to encode a protein of 1,548 amino acids (175 kD) and contained a consensus Kozak sequence, GGCAATGC, at the translation start codon. The Duox2 cDNA sequence is a larger form of a gp91phox homologue identified previously as an NADPH-oxidase in thyroid and termed p138Tox; the latter sequence contained biochemically using the expressed peroxidase domains of human (h)-Duox and Caeorhabditis elegans (Ce)-Duox1.

Results

cDNA cloning of human dual oxidases

The cDNA for h-Duox1 was a 4,563 base pair ORF (sequence data available from GenBank/EMBL/DDBJ under accession no. AF213465) that is predicted to encode a protein of 1,521 amino acids (177 kD), nearly three times the size of gp91phox (Fig. 1). A consensus Kozak sequence, ATG, was present at the translation start codon. The cDNA for h-Duox2 showed a 4,647 base pair ORF (sequence data available from GenBank/EMBL/DDBJ under accession no. AF267981) that is predicted to encode a protein of 1,548 amino acids (175 kD) and contained a consensus Kozak sequence, GGCAATGC, at the translation start codon. The Duox2 cDNA sequence is a larger form of a gp91phox homologue identified previously as an NADPH-oxidase in thyroid and termed p138Tox; the latter sequence contained biochemically using the expressed peroxidase domains of human (h)-Duox and Caeorhabditis elegans (Ce)-Duox1.
a 1,313 amino acid protein. Alignment by homology of the genomic sequences of Ce-Duox1 and Ce-Duox2 identified two new exons 5' of the first predicted exon of Duox2 that were highly homologous to the second and third exons of Duox1 (predicted cDNA structure available upon request), but an exon of Duox2 homologous to exon1 of Duox1 could not be identified by homology. The predicted amino acid sequences of both Ce-Duox1 and Ce-Duox2 show ~30% identity with h-Duox1 and h-Duox2 (Fig. 1 and Fig. 2 A). Ce-Duox1 also contains the same domains as h-Duox1/2 (see below) and is roughly the same size. However, Ce-Duox2 contains a stop codon, which should eliminate the extreme COOH-terminal portion of the protein, which includes a segment of the pyridine nucleotide-binding site. Thus, whereas Ce-Duox2 should contain intact peroxidase and calmodulin-like domains, it is not predicted to encode a functioning NADPH-oxidase domain (Fig. 1). Except for this COOH-terminal region, Ce-Duox2 is 94% identical to Ce-Duox1 at the amino acid level. Both Ce-Duox1 and Ce-Duox2 are located near the end of chromosome I, separated by only 6 kb and in opposite orientations. The high degree of sequence identity and retention of intron structure (un-
published data) and the location of both near the end of a chromosome are consistent with a recent gene duplication.

**Domain organization and sequence comparisons among gp91phox, h-Duox1, Ce-Duox1, and Ce-Duox2**

The domain structure and transmembrane regions in gp91phox, h-Duox1/2, Ce-Duox1, and Ce-Duox2 are diagrammed in Fig. 1. Duox enzymes are homologous to gp91phox in their COOH termini (see http://www.biochem.emory.edu/Lambeth/gp91_homology.pdf for an alignment of these regions). Nox1 (Suh et al., 1999), which is the same size as gp91phox, is more closely related to gp91phox (54% identical) than is the NADPH-oxidase domain of hDuox1 or h-Duox2 (~26% identical to gp91phox). However, h-Duox1 and 2 are more closely related to Ce-Duox1 within the NADPH-oxidase domain (~39% identical). Within the putative flavin adenine dinucleotide (FAD)-binding regions and NADPH-binding regions, homologues share considerably higher homology, ranging from 60 to 90% depending on the region. This includes the canonical dinucleotide-binding helix GXGXXP. In gp91phox, Nox1, h-Duox1, and h-Duox2, this sequence is followed by F, which is present in many NADPH-specific flavoproteins, whereas in the C. elegans proteins F is conservatively replaced with Y.

Duox proteins have additional regions that are not present in gp91phox. A central region contains two EF-hand calcium-binding sequences as indicated in Fig. 1. The canonical residues involved in calcium ligation are well conserved in h-Duox1 and h-Duox2 but are poorly conserved in Ce-Duox1 and Ce-Duox2, suggesting that the function of this region may have evolved away from calcium binding in nematodes.

Surprisingly, the NH2-terminal third of Duox proteins is homologous to peroxidases including MPO, eosinophil peroxidase, thyroid peroxidase, lactoperoxidase, and sea urchin ovoperoxidases (Fig. 2, A and B). Overall, the identity with peroxidases within the entire region is ~20%, but subregions show considerably higher homology. The Duox enzymes represent a distinct group within the peroxidase family (Fig. 2 B), and phylogenetically this group is marginally more closely related to sea urchin ovoperoxidases. Within the peroxidase homology region, only 2 of the 12 cysteine residues involved in the six intrachain disulfide bonds, which are conserved in the four homologous mammalian peroxidases, are present in Duox proteins (Fig. 2 A). In addition, the asparagine-linked glycosylation sites found in MPO are not present in Ce-Duox1 or Ce-Duox2. A calcium-binding site in MPO (aspartate 263 and residues 335–34; Fig. 2 A, superior double bar) (Zeng and Fenna, 1992) is well conserved in the Duox family proteins, including three of the four candidate calcium liganding residues (Fig. 2 A, ▲).

The extreme NH2-terminal 21 amino acids of Ce-Duox1 contain a secretory signal peptide sequence (Fig. 1), implying that the NH2-terminal peroxidase domain is in a compartment that is transmembrane to the cytosol (for example, extracellular or within a secretory vesicle). In addition, hydrophathy plots reveal that the proteins contain a highly hydrophobic region corresponding to the NH2-terminal third of gp91phox. This region can be modeled as a cluster of six transmembrane α helices as indicated in Fig. 1. An additional transmembrane helical region is present between the peroxidase homology domain and the calmodulin-like domain.

**Tissue distribution of h-Duox mRNA**

As shown in Fig. 3, h-Duox1 mRNA was distributed among a variety of adult tissues with highest expression in lung and thyroid but with significant expression also seen in placenta, testis, and prostate, and with detectable expression in pancreas and heart. h-Duox1 mRNA was also widely expressed in fetal tissues where it was abundant in lung. As reported previously (Dupuy et al., 1999), Duox2 (p138H11601) is present in thyroid. In addition, we observed significant expression in a variety of fetal tissues and in adult colon with detectable expression in kidney, liver, lung, pancreas, prostate, and testis.

**Cellular distribution of Ce-Duox1**

The cellular location of Ce-Duox1 was determined by double staining with antibodies to Ce-Duox1 and to the muscle marker myosin A (Fig. 4, A and B). In a longitudinal section, Ce-Duox1 immunostaining was seen in larval animals in a “string-of-pearls” pattern (Fig. 4 A, green) immediately peripheral to muscle (Fig. 4 A, red). Ce-Duox1 (Fig. 4 B, green) was also visualized immediately peripheral to muscle bundles (red) in the cross-sectional plane. Ce-Duox1 staining was considerably weaker in adult animals (unpublished data). The above pattern is consistent with a localization of Ce-Duox1 in hypodermal cells. To verify this localization, double staining was carried out with antibodies to Ce-Duox1 (Fig. 4, C–H, green) and to the hypodermal cell protein MH4 (Fig. 4, C–H, red). Fig. 4, C–E, shows the longitudinal plane, whereas Fig. 4, F and G, shows the cross-sectional plane. Comparison shows that expression of Ce-Duox1 and MH4 occurs in the same cellular pattern, although the staining intensity differed in different regions of the hypodermal cell layer. Merged images are shown in Fig. 4, E and H, and
reveal colocalization in many of the hypodermal cells. Immunostaining with antibody to Ce-Duox1 that had been preincubated with Ce-Duox1(340–355) peptide and antimony A antibodies eliminated staining in the green channel (unpublished data), demonstrating antibody specificity.

**Phenotypes of C. elegans RNAi Ce-Duox animals**

To gain insights regarding the biological function of Duox enzymes, we used the reverse genetic tool, RNAi, to “knock out” Duox in *C. elegans* (Fire et al., 1998). This technique involves injection of double-stranded RNA (dsRNA), encoding a segment of Ce-Duox1 or Ce-Duox2 into gonads of *C. elegans* wild-type hermaphrodites. Injected animals were then allowed to lay eggs, the harvested eggs were allowed to develop, and the progeny were observed for phenotypes. This procedure specifically diminishes or eliminates the expression of the gene of interest. dsRNA corresponding to three distinct regions of Ce-Duox1 and Ce-Duox2 were used in separate experiments. The first two correspond to regions of identity between Ce-Duox1 and Ce-Duox2 and are predicted to block the expression of both forms of Duox. The third dsRNA corresponds to the extreme COOH terminus of Ce-Duox1, which does not have a counterpart in Ce-Duox2, and therefore blocks only the expression of Ce-Duox1. All three dsRNA forms resulted in the expression of Ce-Duox1 and Ce-Duox2 and are predicted to block the expression of Ce-Duox1, which does not have a counterpart in Ce-Duox2, and therefore blocks only the expression of Ce-Duox1. All three dsRNA forms resulted in the expression of Ce-Duox1 in the hypodermal cells. The use of antibody to Ce-Duox1 that had been preincubated with Ce-Duox1(340–355) peptide eliminated green channel antibody staining (unpublished data). Photos are representative of ∼100 animals observed.

Similar phenotypes in *C. elegans* have been described previously and are associated with mutations in the collagen biosynthetic pathway (Levy et al., 1993; Grupta et al., 1997; Johnstone, 2000). Several genes that encode cuticle collagens when mutated result in Bli (“blister”), Dpy (“dumpy,” short fat worm), Rol (“roller,” helical motion instead of a flat, sinuoidal motion), or Sqt (“squat,” generally rollers as larvae and dumpy as adults) phenotypes. The genetics of this process are complex, since for some genes different mutations in the same gene give rise to different phenotypes, and sometimes the phenotypes are combined (for example, “dumpy roller”). In nematodes, collagen along with several other proteins provide the major components of cuticle, an extracellular matrix which acts as an exoskeleton. In a global analysis of expression of all *C. elegans* genes using oligonucleotide arrays (Hill et al., 2000), Ce-Duox1 was expressed at low levels (consistent with its exclusive expression in hypodermal cells) in a stage-specific manner. Expression occurred in a cyclic pattern, peaking during the embryonic stage and at 36 h, corresponding to the peak expression of other genes (Johnstone, 2000) related to collagen/cuticle biosynthesis (*col-14*, *dpy-2, -7, -10*, and *sqt-3*). A second set of collagen/cuticle-related genes (*bli-1, -2, -6, -17, -35, -36, -37, -41, *dpy-13, sqt-1*, and *rol-6, -8*) also show peak expression at 36 h. No significant expression of Ce-Duox2 was seen at any stage. Thus, these data are consistent with a function of Ce-Duox1 in cuticle biogenesis.

The similarity in phenotypes among animals defective in collagen and cuticle biosynthesis compared with the RNAi Duox animals suggested that Duox participates in cuticle biogenesis. To confirm this hypothesis, EM was carried out on wild-type and RNAi animals. As shown in Fig. 6, cuticle of RNAi Duox animals was grossly abnormal. In normal animals (Fig. 6 A), three cuticle layers are...
seen clearly: the cortical (outer), median, and basal (inner) layer as described previously (Cox et al., 1981). The median layer is composed of struts (Fig. 6 A, arrows) connecting the cortical and basal layers with a fluid-filled space between these layers. The RNAi animals (Fig. 6, B and C) frequently showed separation between the cortical and the basal layers with marked expansion of the fluid cavity and broken and distended struts that are still visible on these layers (Fig. 6 B, arrows). These separations occurred mainly over bundles of muscle fiber (Fig. 6, B and C) and are likely to account for the formation of the blisters seen by light microscopy. Thus, the cuticle structure was severely affected in RNAi Duox animals.

**Absence of tyrosine cross-linking in RNAi nematodes**

Cross-linking of collagen and other cuticle proteins in nematodes occurs through di- and trityrosine linkages, which bridge and stabilize the proteinaceous structure (Fetterer and Rhoads, 1990; Fetterer et al., 1993). Because peroxidases such as sea urchin ovoperoxidase and human MPO carry out this reaction (LaBella et al., 1968; Malanik and Ledvina, 1979; Deits et al., 1984), we hypothesized that the function...
of Ce-Duox1 (and possibly Ce-Duox2) is to generate tyrosine cross-links and that the defective cuticle in the Ce-Duox RNAi animals is due to an inability to form tyrosine cross-links. A role for an unknown peroxidase in tyrosine cross-linking in *Ascaris* was suggested previously based on studies in which tyrosine cross-linking activity was inhibited using the peroxidase inhibitors 4-amino-2,3,4 aminotriazole, phenylhydrazine, and N-acetyl tyrosine (Fetterer et al., 1993). We therefore examined the wild-type and Ce-Duox1/2 RNAi knockout animals for di- and trityrosine linkages. An HPLC profile of an acid hydrolysate of the wild-type *C. elegans* is shown in Fig. 7, trace A. The first large peak was identified as dityrosine based on comparison with authentic standard and mass spectral analysis, and the second peak is identified as trityrosine based on its migration on HPLC relative to dityrosine and mass spectral analysis. Based on peak areas and assuming equivalent ionization, dityrosine and tyrosine were present in a ratio of 1:200 in adult wild-type animals. In addition, the fluorescence excitation/emission maxima were determined at alkaline and acidic pH and were in good agreement with previously reported values (Jacob et al., 1996). Mass spectral analysis of purified *C. elegans* cuticle and noncuticular material determined that >99.99% of dityrosine and trityrosine are located in cuticle material, since dityrosine was undetectable in the noncuticle fraction (unpublished data). Dityrosine and trityrosine peaks were absent in hydrolysates of Ce-Duox RNAi nematodes (Fig. 7, trace B). Thus, interference with the expression of Ce-Duox1 eliminates formation of di- and trityrosine linkages.

**Biochemical activities of the expressed peroxidase domains of Ce-Duox1 and h-Duox1**

The peroxidase domains of Ce-Duox (residues 1–590) and h-Duox1 (residues 1–593) were expressed in *E. coli* as described in Materials and methods. A lysate from these cells was analyzed for peroxidase activity, and the results are summarized in Fig. 8 A. As shown, the lysates from *E. coli* expressing both the human and the *C. elegans* peroxidase homology domains from Duox demonstrated peroxidase activity towards 3,3′,5,5′-tetramethylbenzidine (TMB), a well-characterized peroxidase substrate. The activity was inhibited by the peroxidase inhibitor aminobenzohydrazide. Lysates from *E. coli* expressing the peroxidase domains of h-Duox and Ce-Duox but not those from vector control cells also catalyzed the cross-linking of tyrosine ethyl ester (Fig. 8 B). Two major fluorescent products were seen; peak 1 was identified by cochromatography with authentic material as dityrosine, whereas peak 2 was identified as trityrosine by mass spectral analysis as above.

**Discussion**

The topology model shown in Fig. 9 is proposed for Duox based on its primary structure and on analogy with known features of gp91phox. In activated phagocytes, gp91phox is inte-
brane six times (Fig. 1 A), placing both the NH₂ terminus and corresponding region in Duox is predicted to cross the membrane, comprising nearly half of the molecule. The protein domain. Gp91phox contains an NH₂-terminal hydrophobic domain, comprising nearly half of the molecule. The corresponding region in Duox is predicted to cross the membrane six times (Fig. 1 A), placing both the NH₂ terminus and the COOH terminus (attached to the flavoprotein domain) on the cytosolic side. Several features of the model have been verified for gp91phox. For example, the model places known glycosylation sites on the cell exterior (Wallach and Segal, 1997) and a binding loop for the cystosolic regulatory protein p47phox (Biberstine-Kinkade et al., 1999) on the cytosolic side.

The gp91phox homology domains in h-Duox1/2 and Ce-Duox1/2 show the same hydrophyt profile and predicted transmembrane α helices as gp91phox (Fig. 1, hashed bars within the gp91phox homology region). We therefore assume that the gp91phox transmembrane model will also apply to the COOH-terminal portion of the Duox proteins. Such a model predicts that the domain containing the EF-hands is on the interior of the cell. The presence of a secretion export signal peptide sequence at the extreme NH₂ terminus (Fig. 1 A) and the presence of an additional predicted transmembrane hydrophobic sequence intervening between the EF-hand domain and the peroxidase homology domain predicts that the peroxidase domain will reside on the exterior of the cell (Fig. 9). Although features of this model will need to be tested directly, this structure is attractive as it is consistent with the genetic and biochemical data, implicating Ce-Duox1 in the generation of extracellular tyrosine cross-links in cuticle proteins.

The phagocyte NADPH-oxidase serves as a model for the function of the gp91phox homology domain of Duox. The gp91phox component of the phagocyte oxidase generates reactive oxygen outside of the cell or in the phagosome (which is topologically extracellular). NADPH reduces the FAD within the flavoprotein domain, and the FAD then passes electrons through the two heme groups located within the transmembrane NH₂ terminus of gp91phox, reducing oxygen to form superoxide outside of the cell with secondary production of hydrogen peroxide by dismutation. Such a function has been demonstrated for p138Tex (Duox2), which was purified as the hydrogen peroxide–generating NADPH oxidase from thyroid (Dupuy et al., 1999). These authors proposed that p138Tex functions to provide H₂O₂ to thyroid peroxidase, which is known to iodinate the thyroid hormone precursor. A recent study (De Deken et al., 2000) identified a peroxidase homology domain in Duox1 and Duox2 (ThOX1 and ThOX2), but the authors suggested that this domain was inactive based on an absence of putative catalytically important residues.

Oxidative reactions are generally thought to be deleterious to the cell, but the results of the current study suggest that protein oxidation by peroxidases plays a critical role in normal physiology. Insights into the function of the Duox peroxidase domain come from the phagocyte system in which cell activation is accompanied by both activation of the phagocyte NADPH-oxidase and secretion of MPO. Hydrogen peroxide generated indirectly by the phagocyte NADPH-oxidase combines with chloride in an oxidation catalyzed by MPO to form hypochlorous acid, a species which functions in bactericidal reactions. In the case of Duox enzymes, both the NADPH-oxidase moiety and the peroxidase moiety are integrated into a single molecule. The hydrogen peroxide generated by the gp91phox-homology domain in Duox should then serve as a substrate for the peroxidase domain. For Ce-Duox1, the cosubstrate is protein tyrosine residues, which are converted to di- and trityrosine presumably via a reaction involving the tyrosyl radical based on mechanisms established for other well-studied peroxidases; tyrosyl radical recombination would generate di- and trityrosine, resulting in protein cross-linking to stabilize the nematode cuticle. Peroxidases including human MPO (Heinecke et al., 1993) and the sea urchin ovoperoxidase (Deits et al., 1984) catalyze this tyrosine cross-linking, albeit in the former case somewhat inefficiently. Thus, the proposed topological structure of Duox is well suited to support transmembrane peroxidative reaction using intracellular reducing equivalents from NADPH.

Precedent for the involvement of a peroxidase in extracellular matrix structure comes from the fertilization reaction in sea urchin oocytes. Fertilization results in activation of tyrosine cross-linking of extracellular proteins in the oocyte, forming a protective fertilization envelope (Deits et al. 1984). This reaction is catalyzed by ovoperoxidase, a peroxidase secreted by the oocyte. In this system, the hydrogen peroxide is generated by an unknown NADPH-oxidase. Thus, in the case of the sea urchin oocyte individual proteins carry out the peroxide generation and the peroxidative functions respectively. Yeast spore coats also contain dityrosine cross-links that are formed by a heme protein (Briza et al. 1996).

The closest known structural homologues of h-Duox1/2 are Ce-Duox1 and Ce-Duox2. Both h-Duox1/2 and Ce-Duox1 show the same domain structure, including the gp91phox homology domain, the EF-hand domain, and the
peroxidase domain. In the case of h-Duox1/2, the critical calcium-binding residues in the EF-hand domain are well conserved, suggesting a role for calcium in the regulation of the enzyme activity. This has been proposed to account for the calcium dependence for the NADPH-oxidase activity of p138phox (Duox2) (Dupuy et al., 1999). In contrast, the calcium-binding ligands in the EF-hand regions in Ce-Duox1/2 are poorly conserved, suggesting that calcium may not be involved at this site. Calcium-binding regions within the peroxidase domain are well conserved in both Ce-Duox1/2 and h-Duox, suggesting a distinct role for calcium as has been noted for other peroxidases.

The similarity between the peroxidase domain of Ce-Duox1 and h-Duox1/2 raises the possibility that their function will be similar. The peroxidase domains of h-Duox1/2 and Ce-Duox1 are 37% identical to one another, whereas the peroxidase domains of Duox proteins are only 19–20% identical with known mammalian peroxidases. Thus, among known peroxidases or peroxidase domains the h-Duox is most similar to Ce-Duox1, and this may imply similar catalytic with known mammalian peroxidases. Thus, among other than collagen and elastin have not been described, peroxidases.

involved at this site. Calcium-binding regions within the calcium-binding ligands in the EF-hand regions in Ce-Duox1/2 are poorly conserved, suggesting that calcium may not be calcium-binding ligands in the EF-hand regions in Ce-Duox1/2. However, such cross-linking may be nonspecifically associated with a lack of sufficient quantities of material for analysis. In addition, another peroxidase-catalyzed cross-link is formed from the deamination of protein lysyl ε-amino groups to form lysyl aldehydes, which then react with amino acid residues of adjacent molecules (Stahmann et al., 1977; Clark et al., 1986; Hazen et al., 1997). Thus, it is also possible that h-Duox generates this type of extracellular matrix cross-link. Its transmembrane nature and results from RNAi studies in C. elegans support the hypothesis that this enzyme participates in the formation or modification of extracellular protein matrix.

Materials and methods

Cloning of the cDNA for human Duox1

A BLAST search using as a query the NH2-terminal region of a partial clone of Duox2 (see below) identified a 357 base-sequence-enriched portion of an expressed sequence tag (EST, nn00125;1) sequence data available from GenBank/EMBL/DBJ under accession no. AA641653) from an invasive human prostate tumor. The bacterial strain containing the EST sequence in the pBluescript SK-vector was purchased from American Type Culture Collection. The DNA sequenced using T7 and T3 vector primers and sequence-specific primers. The EST insert encoded an ORF of 673 amino acids but lacked both candidate start and stop codons. Northern blot analysis indicated the signal was ~5.5 kb. Rapid amplification of cDNA ends (RACE) and 3′ RACE were carried out using human adult lung mRNA (CLONTECH) as template. 5′ RACE was carried out with a 5′ RACE kit (version 2.0; Gibco BRL) using sequence-specific primers: 5′-GAGAGCTCTGGAGACACTTGAGTTC-3′ and 5′-GAGAGCTCTGGAGACACTTGAGTTC-3′ (for nested PCR). 3′ RACE was carried out with sequence-specific primers: 5′-GAGAGCTCTGGAGACACTTGAGTTC-3′ and 5′-CATGTGTCCTCGTGGTCAAGC-3′ and 5′-CATGTGTCCTCGTGGTCAAGC-3′ (for nested PCR). 5′ RACE yielded 1.1 kb, and 3′ RACE yielded 2.4 kb of new DNA sequence. These procedures generated a sequence, which predicted an ORF of 4,563 base pairs, which encodes a predicted 180-kD protein comprised of 1,521 amino acids.

Cloning of the cDNA for human Duox2

A 535 base portion of an EST (zc92h030.r1) sequence data available from GenBank/EMBL/DBJ under accession no. W32750) from human pancreatic islet was identified using the amino acid sequence of human gp91phox as a query in a BLAST search. The bacterial strain no. 595750 containing the EST sequence in the pBluescript SK-vector was purchased from American Type Culture Collection. The DNA was sequenced using primers to T7 and T3 vector promoters and sequence-specific primers. The EST encoded a 440 amino acid partial cDNA exhibiting 24.4% identity to gp91phox. 5′ and 3′ RACE were carried out using human adult pancreas mRNA (CLONTECH) with the 5′ RACE kit for Rapid...
Amplification of cDNA ends version 2.0 (GIBCO BRL). PCR was done with specific primers: 5′ RACE: primer 1, 5′-GGAAGTTGTGGAGGACCAGAAGCATA-3′; primer 2, 5′-CCCTCGACATTGAGCAGGCTGGTG-3′; primer 3, 5′-CAGCACCACAGATGATCGTGTCAG-3′; primer 4, 5′-GGAAGCGGAGCAAGGACAGTGAAGG-3′; primer 5, 5′-AGGTTGGAGGACCAGGCTGGTG-3′ (for nested PCR); 3′ RACE primer 6, 5′-ACACTGTGCAGGCGAGTCCTTACGA-3′; primer 7, 5′-AGGTTGGAGGACCAGGAGGACGC-3′; primer 8, 5′-TCTCTGACATTGACCTACCTTACGGC-3′ (for nested PCR). To complete the sequence, 5′ RACE was carried out using human thyroid mRNA as a DNA template on CLONTECH with primer 3 and adapter primer AP1 and primer 5 and adapter primer AP2. These procedures resulted in an additional 3.7-kb 5′ region and a 1.5-kb 3′ region.

Identification of genes for Ce-Duoxx1 and Ce-Duoxx2

A BLAST search using the cDNA sequence of human gp91phox identified two putative homologues (sequence data available from GenBank/EMBL/DDJB) under accession nos. AF043697 and AF003130) in the genomic sequence of C. elegans, both near the end of chromosome I and separated by ~6 Kb.

Cloning of the cDNA for Ce-Duoxx1

Based on the gene sequence, PCR primers were designed to amplify two overlapping portions of the Ce-Duoxx1 gene: one extending from the 5′ end and one extending from the 3′ end. Primers were 5′-ATTTGCTCGA-CAAATGCGGTCAAATGCTGGT-3′ and 5′-AATCTTGTGTCATAAAGTGTTGACG-3′ for the 5′ region, and 5′-TGGATTACGATTTTGGTGATG-3′ and 5′-GACCCGCAGGACGAGTTTTTCAAGCAGGACTG-3′ for the 3′ region. PCR was carried out using a random primed C. elegans cDNA library in AACT obtained from R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) under the following conditions: denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. The 5′ piece and the 3′ piece were digested with Dra III and ligated to produce the full-length Ce-Duoxx1 cDNA. The full-length Ce-Duoxx1 cDNA was inserted into the plBluescript SK-vector and was sequenced using T7 and T3 vector primers and sequence-specific primers.

Analysis of primary structure

Export signal sequences were predicted according to Nielsen et al. (1997). C-terminal amphipathic helix was predicted according to Sonnhammer et al. (1998). Both methods are available on the internet at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services). Multiple sequence alignments phylogenetic analysis were carried out using the clustal method using Megalign software (DNASTAR).

PCR detection of mRNA for human Duoxx2

Based on the cloned h-Duoxx1 and hDuoxx2 cDNA sequence, we designed specific primers (Duoxx1: 5′-GCAAGACATACACCGCTACTTCT-3′ and 5′-CTGCACCTACACACAGGGTCCG-3′; Duoxx2: 5′-GCCCTCAACCTCAAG-3′ and 5′-GACCCGCAGGACGAGTTTTTCAAGCAGGACTG-3′), which were used to determine the tissue expression patterns of Duoxx1 and Duoxx2 using human multiple tissue PCR panels and human thyroid gland RNA. PCR was done with Amplification of cDNA ends version 2.0 (GIBCO BRL). The 659-bp fragment generated was digested with SstI and EcoRI and subcloned into pBluescript. For pBluescript.Duoxx2, pBluescript.E17Duoxx1, or pBluescript.E18Duoxx1, exon 10 of Ce-Duoxx2 was amplified by PCR from genomic DNA using the forward primer 5′-CGGTATTACGATTTTGGTGATG-3′ and the reverse primer 5′-CAGCTCACAACTG-3′. The 266-bp fragment generated was digested with SstI and EcoRI and subcloned into pBluescript. Dityrosine standard was synthesized and purified as in Abdelrahim et al. (1997). Goat anti–mouse rhodamine-conjugated antibody and goat anti–rabbit FITC-conjugated antibody were used as secondary antibodies for the detection of Ce-Duoxx1 antibody, myosin A antibody, and MH4 antibody. Mouse antibody to myosin A was a gift from D. Miller (Vanderbilt University, Nashville, TN) (Miller et al., 1983). The MH4 monoclonal antibody developed by G.R. Francis and R.H. Waterston (Washington University, St. Louis, MO) (Francis and Waterston, 1991) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and distributed by The University of Iowa, Department of Biological Sciences. To determine the non-specific binding of the Ce-Duoxx1 antibody, a 10-fold molar excess of Ce-Duoxx1(340–355) peptide was added to neutralize the antibody. Microscopy was carried out using a Zeiss 510 laser scanning confocal microscope.

Preparation of dityrosine standard

Dityrosine standard was synthesized and purified as in Abdelrahim et al. (1997) with minor modifications. Reaction products were dissolved in acidified methanol, filtered, and directly applied to the CP-11 cellulose phosphate, eliminating the rotary evaporation step. Samples with absorption properties characteristic of dityrosine were pooled and freeze dried. For mass spectrometry, the dityrosine standard (0.77 mg/ml) was added to 1 ml of methanol–water (1:1) in 0.1% acetic acid.

Analysis of dityrosine and trityrosine

Nematodes were washed with M9 buffer, suspended in 0.5 ml sonication buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride), and sonicated four times for 20 s. Protein was determined with the Bradford assay using BSA as a standard. 10 μg of whole animal extract was loaded onto a 10% SDS-page gel, which was then transferred to Immobilon-P membrane (Millipore). The blot was blocked for 1 h in a solution of 5% nonfat powdered milk and 0.05% Tween in PBS. The antibody to Ce-Duoxx1 was added in a 1 to 2,000 dilution, incubated overnight, and the membrane was washed three times for 15 min with blocking solution. The blot was then developed using a SuperSignal chemiluminescent kit from Pierce Chemical Co. A Western blot of C. elegans protein extract showed a single band with a molecular weight of ~180,000 (unpublished data).

Indirect immunofluorescence

Immunofluorescence staining of C. elegans was carried out as in Benjam et al. (1996). Goat anti–mouse rhodamine-conjugated antibody and goat anti–rabbit FITC-conjugated antibody were used as secondary antibodies for the detection of Ce-Duoxx1 antibody, myosin A antibody, and MH4 antibody. Mouse antibody to myosin A was a gift from D. Miller (Vanderbilt University, Nashville, TN) (Miller et al., 1983). The MH4 monoclonal antibody developed by G.R. Francis and R.H. Waterston (Washington University, St. Louis, MO) (Francis and Waterston, 1991) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and distributed by The University of Iowa, Department of Biological Sciences. To determine the non-specific binding of the Ce-Duoxx1 antibody, a 10-fold molar excess of Ce-Duoxx1(340–355) peptide was added to neutralize the antibody. Microscopy was carried out using a Zeiss 510 laser scanning confocal microscope.

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Analysis of dityrosine and trityrosine

Nematodes were washed with M9 buffer, suspended in 0.5 ml sonication buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride), and sonicated four times for 20 s. Protein was determined with the Bradford assay using BSA as a standard. Whole worm extracts were lyophilized and resuspended in 6 N HCl. Samples were hydrolyzed for 24 h at 110°C under vacuum, dried under vacuum, and resuspended in the mobile phase for analysis by HPLC on a C18 column (0.46 x 26 cm; Fisher Scientific) using a Dionex Apx-1 HPLC instrument. The mobile phase consisted of 0.1 M KH2PO4 adjusted to pH 3.8 with 0.1 M phosphoric acid at a flow rate of 1 ml/min. The column eluent was monitored by fluorescence with an excitation 305–395-nm bandpass filter and an emission filter at 450 nm with a Bandpass 400 nm detection wavelength. To verify the identity of dityrosine, authentic dityrosine standard was added to some samples and an increase in the intensity of the putative dityrosine band was observed (unpublished data).
Spectroscopic properties of di- and trityrosine

HPLC-purified samples of dityrosine and trityrosine from both C. elegans extracts and peroxidase domain cross-linking reactions were lyophilized and resolubilized in either 0.1 M HCl (3 ml) or 0.1 M NaOH (3 ml). Fluorescence excitation and emission spectra were obtained with a PerkinElmer LS-5B luminescence spectrometer.

Mass spectrometry

Mass spectrometry was performed on a PerkinElmer scieX API 3000 triple quadrupole mass spectrometer equipped with a turboionspray source. Dried dityrosine standard (20 mg) was reconstituted in 200 μl of H2O. A 50-μl aliquot of this was diluted to a final volume of 1 ml with 950 μl of 5 mM ammonium acetate in MeOH and 1% acetic acid. This solution was infused at a flow rate of 5 μl min⁻¹. The ionspray needle was held at +550 and −4500 V for positive and negative ion analysis, respectively. These experiments identified the singly protonated (positive ion mode) and deprotonated (negative ion mode) species of the standard to be m/z 361.3 and 359.3, respectively, corresponding to prediction.

Total protein, purified cuticle, and total protein minus purified cuticle acid hydrolysates from C. elegans, and standard were analyzed by reverse phase LC-MS/MS. A 50-μl volume of sample was injected onto a 15 cm × 2.1 nm Supelco Discovery C18 column eluted at a flow rate of 380 μl min⁻¹. Solvent A was 99:1 H₂O to acetic acid and solvent B was 99:1 MeOH:acetic acid, both containing 5 mM ammonium acetate. The column was infused directly into the ion source of the mass spectrometer operating in positive ion mode. The column was preequilibrated with 100% solvent A for 6 min followed by sample injection. The column was then washed with 100% solvent A for 4 min and eluted with a 1 min linear gradient to 100% solvent B followed by a 4 min wash with 100% solvent B. For these experiments, both the precursor ions (as above for dityrosine; m/z 540.4/538.4 for trityrosine) and structurally distinctive breakdown ions were monitored. The transitions monitored for dityrosine were the neutral loss of both COOH groups and both NH₂ groups (m/z 269.4, 252.2, and 235.0, respectively). For trityrosine, the transitions monitored were the neutral loss of two COOH groups, the neutral loss of a COOH group and one NH₂ group, the neutral loss of two COOH-termini, and the neutral loss of two COOH groups and two NH₂ groups (m/z 494.3, 477.2, 448.2, and 431.2, respectively). C. elegans cuticle was purified according to methods developed previously by Cox et al. (1981).

Transmission electron microscopy

Approximately 120 wild-type or RNAi-blistered adult C. elegans were collected and washed first with M9 buffer and then with 0.1 M caccodylate buffer (pH 7.4). Animals were pelleted, added to 1 ml of 0.8% glutaraldehyde, 0.1% m-caccodylate, pH 7.4, and incubated on ice for 1.5 h with occasional mixing. The animals were washed with 0.1 M caccodylate buffer, transferred to a glass depression slide, and fixed using 2% glutaraldehyde, 0.1 M cacodylate buffer, transferred to a glass depression slide, and fixed using 2% glutaraldehyde, 0.1 M cacodylate buffer, and 0.1 M cacodylate buffer, and 0.1 M cacodylate buffer, and 0.1 M cacodylate buffer, and 0.1 M cacodylate buffer, and stained with uranyl acetate and lead citrate, and cross sections were examined with a Philips EM201 electron microscope.

Construction of Duox peroxidase domain expression plasmids

The PCR was used to amplify the peroxidase domains of h-Duox (amino acid residues 1–593) and Ce-Duox (amino acid residues 1–590) from the cloned full-length sequences. The primers were designed to introduce an NH₂-terminal BamH I site and a COOH-terminal Not I site. PCR products were digested with BamH I and Not I and ligated into the pET-32(a) + vector from Novagen. Plasmids were transformed into BL21(DE3) cells containing the chloramphenicol-resistant plasmid pT-gro (Yasukawa et al., 1995), which expresses the chaperonins groES and groEL from the T7 promoter. The pT-gro expression vector in BL21(DE3) cells was a gift from Dr. Lee-Ho Wang (University of Texas Health Science Center, Houston, TX) and Dr. Shunzuke Ishii (Institute of Physical and Chemical Research, Ibaraki, Japan). LB-agar plates containing both ampicillin and chloramphenicol were used to isolate colonies.

Expression of Duox peroxidase domains

A 0.5-ml LB overnight culture of cells containing plasmid with the peroxidase domain from h-Duox or Ce-Duox was used to inoculate 50 ml of modified TB medium (Sandhu et al., 1993) containing 0.5 mM β-amino- phenylpropionic acid, 100 μg/ml ampicillin, and 25 μg/ml chloramphenicol in a 250-ml flask. Bacteria were grown at 37°C in a shaker at 200 RPM until the cell density measured 0.7 OD at 600 nm. Isopropyl-β-D-thiogalactopyranoside (1 mM) was added, and the culture was continued at 25°C for 24 h, and at 150 RPM. Cells were pelleted at 4,500 g and resuspended in PBS containing 4-[2-aminoethyl]benzenesufluyl fluoride (2 μM), bestatin (130 mM), trans-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane (1.4 mM), leupeptin (1 nM), and aprotinin (0.3 nM). The cell suspension was then sonicated on ice.

Activity assays

The TMB liquid substrate system (Sigma-Aldrich) was used to assay peroxidase activity (Holland et al., 1974). 100 μg of lysate protein from cells expressing either the human Duox1 peroxidase domain, Ce-Duox1 peroxidase domain, or a vector control was added to 1-ml aliquots of the TMB substrate system. The peroxidase reactions were performed in triplicate, and activity was monitored at 655 nm with a Beckman Coulter DU640B spectrophotometer. Some samples contained 30 μM aminobenzenoic acid hydrazide, a peroxidase inhibitor (Kettle et al., 1995). To assay tyrosine cross-linking, tyrosine ethyl ester (20 mM) was dissolved in 10 ml of PBS buffer supplemented with 80 μl of 3% H₂O₂. 100 μg of E. coli lysate protein was added to 1-ml aliquots, samples were incubated for 1 h, and the reaction was quenched using an equal volume of 12 M HCl. Samples were analyzed for di- and trityrosine as above.

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