Novel homologs of gp91*phox*

Reactive oxygen species (ROS) are cytotoxic and mutagenic, as they modify and damage critical biomolecules including DNA and lipids. This property of ROS is exploited by phagocytes, which generate large amounts of superoxide and hydrogen peroxide (H_2O_2) as part of their armory of bactericidal mechanisms[1]. The occurrence of ROS in non-phagocytic cells has been documented over the past decade[2,3], but its significance has been debated. ROS in these cells is often considered an 'accidental' byproduct of metabolism, particularly mitochondrial respiration[4]. Evidence points to such accidental ROS generation in the cumulative somatic-cell genetic changes seen in aging[5].

Recent studies imply more specific roles for reactive oxygen. Exposure of cells to the growth factors platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) induces the production of H_2O_2, which activates components of signalling pathways including p42/p44 MAPK, and tyrosine phosphorylation[6,7]. Mutationally activated Ras stimulates superoxide generation[8]. ROS are also generated by specialized cells and are implicated in their distinct functions. For example, osteoclasts generate superoxide, which participates in bone resorption[9]. An H_2O_2-generating enzyme in sea urchin eggs plays a role in hardening of the fertilization membrane[10], and H_2O_2 generated in thyroid cells functions in the biosynthesis of thyroid hormone[11].

In the phagocyte, reactive oxygen generation is catalysed by an NADPH-oxidase. gp91*phox* is the catalytic moiety of the phagocyte NADPH-oxidase, a plasma membrane-associated flavohemoprotein complex containing one flavin-adenine dinucleotide (FAD) and two hemes that catalyses the NADPH-dependent reduction of O_2 to form superoxide[12]. In resting phagocytes, the enzyme is dormant but becomes activated by assembly with the regulatory proteins p47*phox*, p67*phox* and Rac[13]. This article chronicles the discovery and initial characterization of eukaryotic homologs of gp91*phox*.

New homologs of gp91*phox*

The cDNAs for five new human homologs of gp91*phox* have been cloned, and additional homologs have been identified in rodents, *C. elegans* and *Drosophila* (Table 1). cDNA or genomic sequences, or both, for each are available in GenBank. The region in each of these proteins that is homologous to gp91*phox* has been aligned (http://www.biochem.emory.edu/Lambeth/gp91_homology.pdf). Evolutionary relationships based on this alignment (Fig. 1) define three groups: a gp91*phox*-like group (Nox1, 2 and 3), Nox4 and the Duox group. Nox1–4 proteins are 65–66 kDa, whereas the Duox proteins range from 175 to 180 kDa. The first of the homologs reported[14] was Mox1 (or mitogenic oxidase). Because the function of other homologs has not been described, and because Mox has been used to name other genes, these are now termed Nox (for NADPH-oxidase). We refer to the large homologs as Duox (for dual oxidase), as explained below. Both terminologies have been accepted by the HUGO Human Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/), and parallel terminologies are recommended for homologs in other mammalian species.

Sequence comparisons with gp91*phox*

Homologous domains in the Nox and Duox groups are shown in Fig. 2a. For the Nox group proteins, the entire molecule is homologous to gp91*phox*. The C-terminal half of the gp91*phox* homology region is homologous to known flavoproteins. Highly conserved subregions generally correspond to FAD- and NADPH-binding sites (http://www.biochem.emory.edu/Lambeth/gp91_homology.pdf). Residues 410–415 in gp91*phox* match the canonical nucleotide-binding sequence Gly-Xaa-Gly-Xaa-Xaa-Pro. In flavoproteins

Table 1. Eukaryotic homologs of gp91*phox*

Name, alias	Species	GenBank no.[a] (cDNA)	Gene name/locus	GenBank no. (genomic DNA)	Protein size (kDa)	Ref.
gp91*phox*, Nox2	Human	NM_000397	CYBB/Xp21.1		65.3	21
	Mouse	U43384			65.3	22
	Bovine	AF036097			65.6	23
Mox, Nox1, NOH-1	Human	AF127763	NOX1/Xq22	HS146H21	64.9	14
	Rat	AF152963			65.2	14
Nox3	Human	AF190122	NOX3/6q25.1-26	HS257I9	64.9	b
Nox4	Human	AF254621	NOX4/11	AC023784	66.9	b
	Mouse	NM_015760			66.5	c
p138[Tox], Duox2	Human	AF181972[d]	DUOX2/15q21	AC009700	175.4	15
		AF267981				b
	Pig	AF181973				15
Duox1	Human	AF213465	DUOX1/15q21	AC009700	177.3	b
Ce-Duox1	*C. elegans*	AF229855	Chromosome 1	AF043697	170.4	b
Ce-Duox2	*C. elegans*		Chromosome 1	AF003139	150	b
Duox	*Drosophila*		2L (23B2-23C1)	AE003582	170.8	b

[a]Most of the homologs were identified by BLAST searching using the gp91*phox* sequence as a query. This permitted the identification of expressed sequence tagged (EST) clones containing partial sequences, which were then completed using 5′- and 3′-RACE. Nox3, Ce-duox1 and 2 and *Drosophila* Duox, the new homolog, were first predicted from genomic sequences available in public databases.
[b]G. Cheng, Z. Cao, W.A. Edens, L. Sharling, T. Lee, R.S. Arnold and D. Lambeth, submitted to GenBank.
[c]S. Yang and L. Key, Jr, submitted to GenBank.
[d]Incomplete cDNA sequence.

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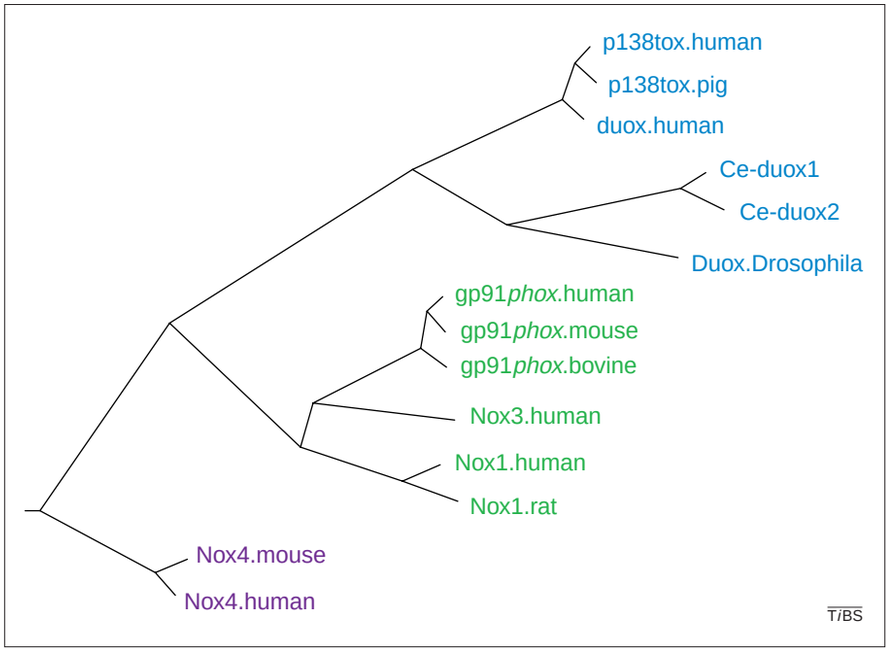

Figure 1

Dendogram based on amino acid sequence comparisons among gp91*phox* homologs. Sequences within the regions homologous to gp91*phox* were aligned (http://www.biochem.emory.edu/Lambeth/gp91_homology.pdf) with Megalign using the Clustal method, and the alignment was used to construct the dendrogram showing sequence distances. Percent identities ranged from 17% to 60% for homolog pairs. Nox1 is also known as Mox1.

that bind NADP(H), this is followed by Phe, whereas in many NAD(H)-flavoproteins, this is followed by Met. In 12 of the 14 homologs in Table 1, this residue is Phe and is conservatively replaced with Tyr in two, predicting that all members will prefer NADPH.

In Nox1–4, the N-terminal half of the molecules contain a cluster of five hydrophobic segments that are predicted to be transmembrane α helices (Fig. 2a; for algorithms, see http://genome.cbs.dtu.dk). Their extreme N termini are also hydrophobic but are predicted to be secretion signal peptides that would be absent in the mature protein. Five strictly conserved histidines fall within or are located very close to the transmembrane helices, and four of these should participate in heme ligation.

High-molecular-weight homologs of gp91*phox*

Large homologs of gp91*phox* have been identified in human, *C. elegans* and *Drosophila* (see Table 1). These encode a C-terminal region that is homologous to gp91*phox* (Fig. 2a), except that they contain one additional transmembrane α helix in the transmembrane cluster. A central domain contains two regions that are predicted to be EF-hands, a motif that contains a calcium-binding site, as in calmodulin. An N-terminal region is homologous to peroxidases, including myeloperoxidase. Hence, we term this group of enzymes Duox or dual oxidases, because they contain both an NADPH-oxidase and a peroxidase-homology region. $p138^{Tox}$ is closely related to human Duox1 but lacks the peroxidase-homology domain[15]. However, the translation start site was initially misidentified, and the cDNA actually encodes a larger protein that includes the peroxidase domain (hence, we prefer the name Duox2).

Topology model for Nox and Duox

In the topology model for Nox1–4 (Fig. 2b, left) the two hemes are bound within the transmembrane cluster, and the flavoprotein domain is inside the cell. The physical arrangement of the prosthetic groups predicts that the electron flow is as follows: cytosolic NADPH → FAD → heme1 → heme2 → oxygen, forming a superoxide transmembrane to the cytosolic flavoprotein domain. In the case of the phagocyte NADPH-oxidase, ROS are produced outside the cell or in the phagosome. The membrane location of the small homologs of gp91*phox* has not been reported, but extracellular superoxide dismutase partially blocks reduction of nitroblue tetrazolium (NBT) in Nox1-transfected cells[14], suggesting that some Nox1 is located in the plasma membrane.

The predicted topology for the Duox homologs within the gp91*phox*-homology domain (Fig. 2b, right) is similar to that of gp91*phox*, except that the additional transmembrane helix recrosses the membrane, placing the EF-hand domain on the cytosolic side. The peroxidase domain contains a secretion export signal, localizing this domain transmembrane to the cytosol. One additional transmembrane helix spans the membrane between the peroxidase domain and the EF-hand region.

Tissue expression of homologs of gp91*phox*

In contrast to gp91*phox*, the Nox and Duox mRNAs that have been examined are not expressed in inflammatory cells. Instead, each shows a distinct tissue expression pattern: for example, the mRNA for Nox1 is highly expressed in colon epithelial cells with lower levels in the prostate, uterus and vascular smooth muscle cells, where it is induced by PDGF (Ref. 14). $p138^{Tox}$ is highly expressed in thyroid cells[15].

Functions of Nox1

Nox1 functions in mitogenic regulation and cell transformation[14]. In vascular smooth muscle, decreased expression of endogenous Nox1 caused by antisense DNA correlated with decreased ROS level and decreased growth, indicating a role for Nox1 in normal growth. Surprisingly, fibroblasts overexpressing Nox1 showed increased mitogenic rates and a transformed appearance. These cells were highly tumorigenic in athymic mice, producing sizable tumors within three weeks. Although it is not currently known whether Nox enzymes play a role in human cancers, it is intriguing that increased ROS levels are observed in cancer cells[16]. Reactive oxygen is also proposed to contribute to the origin of cardiovascular disease[17]. An unidentified pyridine nucleotide-dependent superoxide-generating activity in vascular smooth muscle has been linked to increased cellular ROS in models of atherosclerosis and hypertension[18], and Nox1 now appears to account for this activity[14].

Nox1 is expressed in alternatively spliced forms, including a small form referred to as NOH-1S, which is predicted to encode a 191-amino-acid protein corresponding to the N terminus of Nox1 (Ref. 19). This form encodes four of the transmembrane helices but lacks the flavoprotein domain and essential heme-binding histidines. Hence, its function does not involve the generation of reactive oxygen. Rather, overexpressed

NOH-1S showed properties indistinguishable from a voltage-gated H^+ channel. It is not known whether other Nox-family members also have alternative splice forms that function in proton transport.

Function of Duox enzymes

p138^{Tox} has been identified as the NADPH-oxidase that supports thyroid hormone biosynthesis[15]. The synthesis of thyroid hormone involves iodination of thyroglobulin, a reaction that is catalysed by thyroid peroxidase and an H_2O_2-generating NADPH-oxidase. The oxidase was purified based on its NADPH-dependent diaphorase activity, and peptide microsequencing provided the key to cloning p138^{Tox}. The presence of EF-hand motifs could account for the calcium dependency of the thyroid NADPH-oxidase. However, the function of the peroxidase domain is unclear, and its peroxidase activity remains to be proven. Iodination of thyroglobulin is one possibility, although this would imply that the function of the peroxidase domain duplicates the well-characterized function of thyroid peroxidase[20]. The function of the other members of the Duox group also remains to be established. Their topology (Fig. 2b) suggests a general function in transmembrane peroxidative reactions in which the NADPH-oxidase moiety functions to generate superoxide that dismutes to form the H_2O_2 substrate for the peroxidase domain. The reaction catalysed will depend on the specificity of the peroxidase domain and the presence of peroxidizable substrates, and could include halogenations, tyrosine crosslinking or other oxidations.

Concluding remarks

The existence of homologs of gp91*phox* in noninflammatory tissues implies that the generation of reactive oxygen in these tissues is not an accident of respiration but is a deliberate biological strategy. The recent cloning of multiple homologs of gp91*phox*, as well as intriguing recent studies regarding the biochemical and cellular functions of some of these homologs, provide a starting point for understanding the biological relevance of this new group of enzymes.

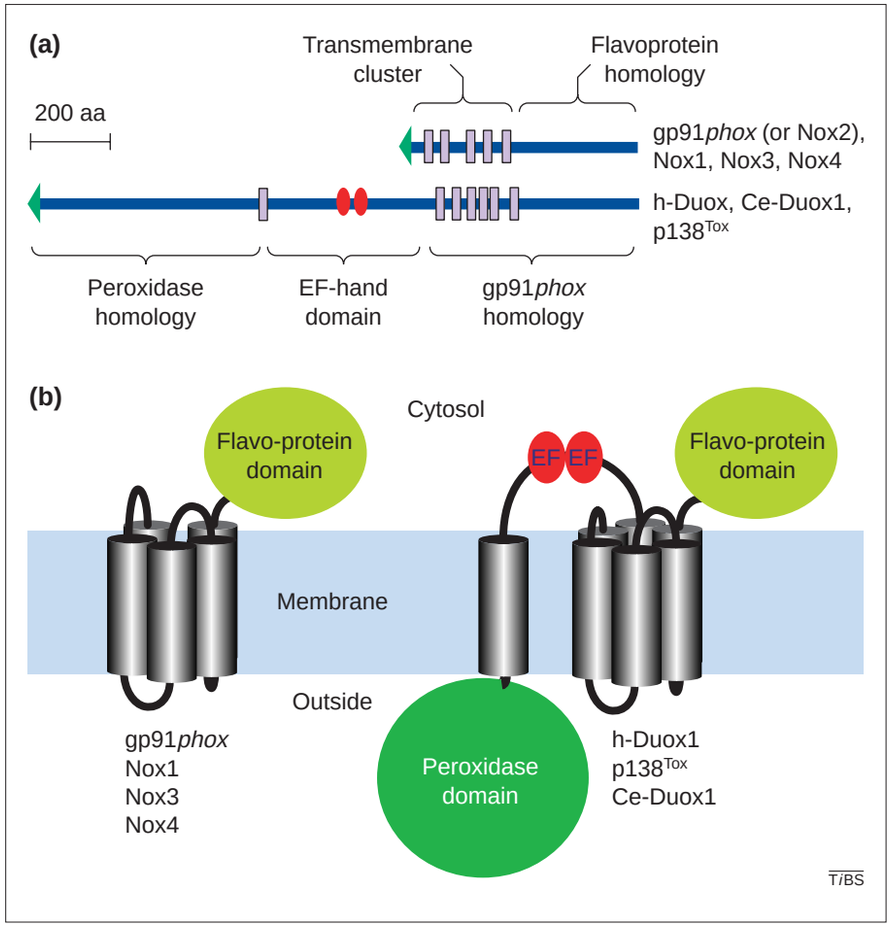

Figure 2

Domain structure and topology model of Nox and Duox enzymes. **(a)** Domains that are homologous to gp91*phox* and to peroxidases, and a domain containing two EF-hand motifs (red circles). A flavoprotein homology region and a transmembrane cluster are also indicated (light-purple boxes are predicted transmembrane α helices). The green triangles indicate predicted secretion export signal peptides. **(b)** The topology model for Duox and Nox enzymes is based on predicted transmembrane α helices and the presence of predicted export signal peptide sequences.

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