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Review

Molecular composition and regulation of the Nox family NAD(P)H oxidases ☆

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Abstract

Reactive oxygen species (ROS) are conventionally regarded as inevitable deleterious by-products in aerobic metabolism with a few exceptions such as their significant role in host defense. The phagocyte NADPH oxidase, dormant in resting cells, becomes activated during phagocytosis to deliberately produce superoxide, a precursor of other microbicidal ROS, thereby playing a crucial role in killing pathogens. The catalytic center of this oxidase is the membrane-integrated protein gp91^{phox}, tightly complexed with p22^{phox}, and its activation requires the association with p47^{phox}, p67^{phox}, and the small GTPase Rac, which normally reside in the cytoplasm. Since recent discovery of non-phagocytic gp91^{phox}-related enzymes of the NAD(P)H oxidase (Nox) family—seven homologues identified in humans—deliberate ROS production has been increasingly recognized as important components of various cellular events. Here, we describe a current view on the molecular composition and post-translational regulation of Nox-family oxidases in animals.

Keywords: ROS; Nox; Duox; gp91^{phox}; p22^{phox}; p47^{phox}; p67^{phox}; Noxo1; Noxa1; Rac

Reactive oxygen species (ROS) are classically described as harmful by-products in aerobic metabolism. On the other hand, there exist enzymes dedicated to ROS production, the first example of which is an NAD(P)H oxidase (Nox) expressed in professional phagocytes such as neutrophils and monocytes/macrophages [1–4]. The phagocyte NADPH oxidase is dormant in resting cells. During phagocytosis of invading micorobes, this enzyme becomes activated to produce superoxide anion (O_2^-) with the secondary generation of other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), and hypochlorous acid (HOCl), which together function in microbial killing in the phagosome (Fig. 1). The importance of the phagocyte oxidase in host defense is evident; recurrent and life-threatening infections occur in patients with chronic granulomatous disease (CGD) whose phagocytes geneticallly lack the superoxide-producing activity [1–4].

The active phagocyte oxidase is a multi-protein complex, in which the membrane-integrated protein gp91^{phox} (otherwise known as Nox2), tightly associated with p22^{phox}, harbors all redox components from NADPH to molecular oxygen for superoxide production (Fig. 2A). The fully assembled complex also contains some modular regulatory proteins (p47^{phox}, p67^{phox}, and p40^{phox}) and the small GTPase Rac, all of which reside in the cytoplasm prior to cell stimulation (Fig. 2B). Recent expansion of information available in genome databases has led to identification of several novel homologues of gp91^{phox} in animals, which constitute the Nox family [5–8]. The human genome contains seven members: Nox1 through Nox5,

☆ *Abbreviations:* ROS, reactive oxygen species; Nox, NAD(P)H oxidase; CGD, chronic granulomatous disease; PRR, proline-rich region; AIR, autoinhibitory region; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; TPR, tetratricopeptide repeat; Noxo1, Nox organizer 1; Noxa1, Nox activator 1; RNAi, RNA interference; MPO, myeloperoxidase; TPO, thyroid peroxidase.

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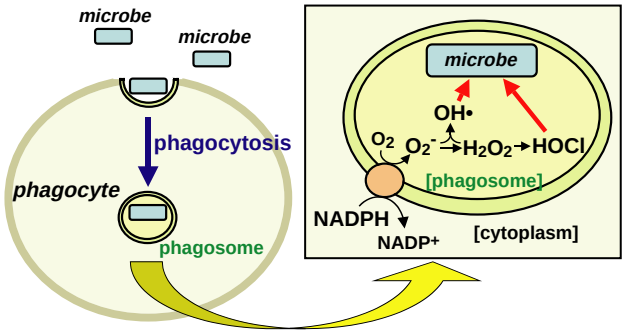

Fig. 1. Oxygen-dependent killing of microbes by phagocytes. During phagocytosis of invading microbes, the phagocyte NADPH oxidase becomes activated to produce superoxide (O_2^-), a precursor of microbicidal ROS such as H_2O_2, $OH^\cdot$, and HOCl.

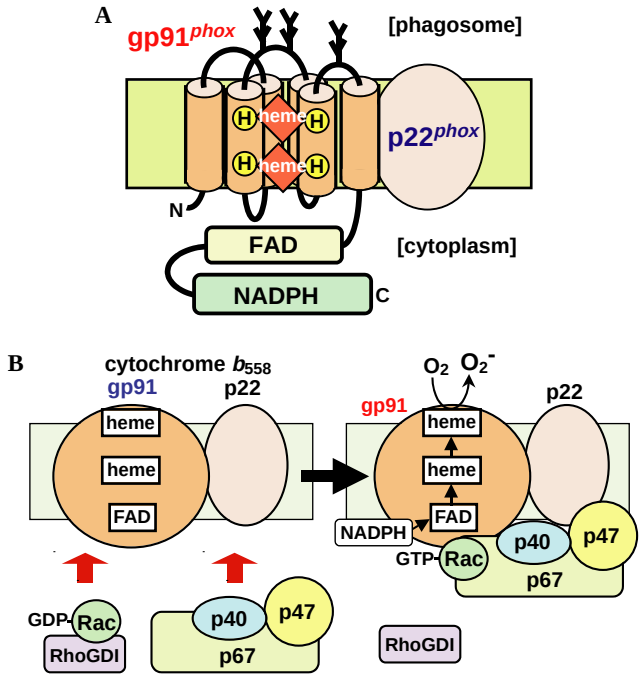

Fig. 2. (A) Structure of gp91^{phox}/Nox2 complexed with p22^{phox}. (B) Formation of the fully assembled, active complex of the phagocyte NADPH oxidase.

and the dual oxidases Duox1 and Duox2. It is currently known that, besides animals, ROS-producing NAD(P)H oxidases, distantly related to animal Nox enzymes, exist in a variety of eukaryotes including plants [9], fungi [10], and the myxomycete (Mycetozoa) *Dictyostelium discoideum* [11]. Thus, deliberate ROS production by these oxidases has been increasingly recognized as important components of various biological events, including hormone biosynthesis and cell signaling, in addition to well-established roles in host defense [5–8]. In this review, we focus on the molecular composition and post-translational regulation of the Nox-family oxidases, with special attention to roles of p22^{phox}, p47^{phox}, p67^{phox}, Rac, and their homologous proteins.

Regulation of the phagocyte NADPH oxidase gp91^{phox}/Nox2

The catalytic subunit of the phagocyte oxidase, gp91^{phox}/Nox2, is a highly glycosylated membrane-spanning protein. The N-terminal portion of gp91^{phox} contains six transmembrane α-helices: the third and fifth helices each contain two invariant histidine residues that are positioned to coordinate two hemes, thereby placing one heme toward the inner face and the other toward the outer face (Fig. 2A). On the other hand, the C-terminal half of gp91^{phox} folds into a cytoplasmic domain containing the FAD- and NADPH-binding sites that exhibit distant but recognizable homology to the ferredoxin reductase family [1–4]. Hence, gp91^{phox} contains the entire transmembrane redox machinery, in which electrons are transferred from NADPH on the cytoplasmic side via FAD and two hemes to molecular oxygen in the extracellular or intraphagosomal space.

Since inappropriate or excessive production of ROS results in inflammatory disorders, the activity of the phagocyte oxidase should be strictly regulated. In resting cells, gp91^{phox} does not produce superoxide. Activation of gp91^{phox}, i.e., successful electron transfer in gp91^{phox}, requires stimulus-induced membrane translocation of p47^{phox}, p67^{phox}, and Rac (Fig. 2B). The essential role of these regulatory proteins becomes evident by the following two lines of evidence. First, the phagocyte NADPH oxidase activity can be reconstituted in a cell-free system with gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and Rac, using an anionic amphiphile, e.g., arachidonic acid, as an in vitro stimulant. Second, all but one patient with CGD, to date, are known to have defect of one of the genes for gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} [1–4], the exception being an unfortunate individual with a complex phenotype reflecting an inhibitory (dominant negative) mutation in the Rac2 gene [12]; Rac2 is the predominant Rac isoform in human neutrophils, while Rac1 and Rac2 are both expressed in monocytes/macrophages.

p22^{phox} is a non-glycosylated integral membrane protein, containing two (or possibly four) transmembrane segments, and forms a mutually stabilizing complex with gp91^{phox}, termed cytochrome b_{558} (Fig. 2). During synthesis of the cytochrome, heme incorporation into gp91^{phox} is essential for heterodimer formation, which in turn precedes glycosylation of gp91^{phox} [13]. The C-terminal cytoplasmic tail of p22^{phox} contains a proline-rich region (PRR), which serves as an anchoring site for p47^{phox}, thereby juxtaposing gp91^{phox} and the cytosolic regulatory proteins for formation of the active oxidase complex. Thus, p22^{phox} is crucial for regulation of gp91^{phox}.

The regulatory adaptor protein p47^{phox} harbors tandem SH3 domains (Fig. 3A). Although SH3 domain is known as a module for protein–protein interaction, the two SH3 domains of p47^{phox} are incapable of binding to target proteins in a resting state. This is because they are normally masked via an intramolecular interaction with an

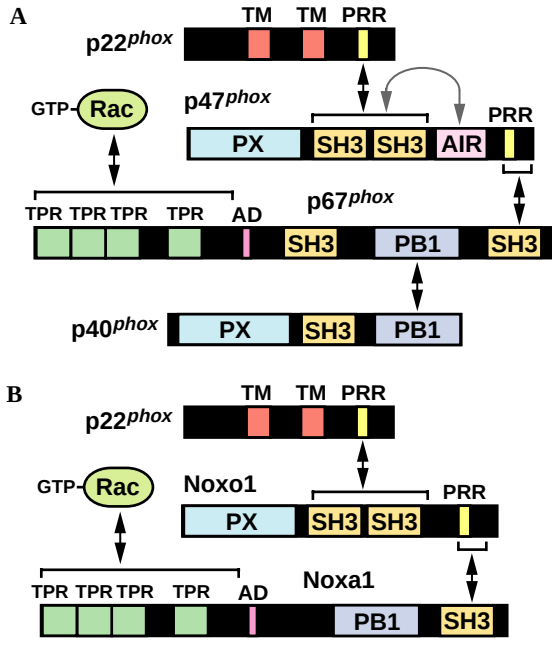

Fig. 3. Interactions of $p22^{phox}$ and Rac with classical (A) and novel (B) regulatory proteins. TM, transmembrane segment; PRR, proline-rich region; AIR, autoinhibitory region; TPR, tetratricopeptide repeat; AD, activation domain.

autoinhibitory region (AIR) that exists C-terminal to the SH3 domains [14–16]. In addition to the two SH3 domains, $p47^{phox}$ harbors a phosphoinositide-interacting PX domain in the N-terminus [17]. The $p47^{phox}$ PX domain binds to phosphoinositides such as phosphatidylinositol 3,4-bisphosphate, albeit with a relatively low specificity and affinity [18,19], and its lipid-binding activity is also negatively regulated under resting conditions [20]. During phagocytosis or with appropriate stimuli such as phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC), $p47^{phox}$ undergoes phosphorylation at multiple serines, several of which exist in the AIR [21]. The phosphorylation of the AIR, in cooperation with other agonists such as arachidonic acid [22], induces a conformational change of $p47^{phox}$ to render both SH3 and PX domains in a state accessible to their own targets: the former binds to the C-terminal PRR of the cytochrome b_{558} subunit $p22^{phox}$, while the latter interacts with membrane phosphoinositides. Cooperation of these two interactions, each being indispensable, allows $p47^{phox}$ to be targeted to cytochrome b_{558}, which is crucial for phagocyte oxidase activation [20].

Another oxidase-specific regulatory protein, $p67^{phox}$, is composed of the following protein-binding modules: an N-terminal domain comprising four tetratricopeptide repeat (TPR) motifs, the central and C-terminal SH3 domains, and a PB1 domain between the two SH3 domains (Fig. 3A). Cell stimulant-triggered recruitment of $p67^{phox}$ to the membrane is dependent on its binding to $p47^{phox}$, which is mediated via a tail–to–tail interaction between the $p67^{phox}$ C-terminal SH3 domain and the $p47^{phox}$ C-terminal PRR [23]. The membrane-associated $p67^{phox}$ functions together with Rac to activate the oxidase, as described below.

Under resting conditions, Rac forms a heterodimeric complex with the inhibitory protein RhoGDI in the cytoplasm. Upon phagocyte stimulation, Rac is released from the complex and recruited to the membrane in a manner independent of $p47^{phox}$ and $p67^{phox}$. At the membrane, Rac is converted to the GTP-bound active form via the function of guanine nucleotide releasing factor for Rac. Active Rac interacts with $p67^{phox}$ via binding to the N-terminal tetratricopeptide repeat (TPR) domain, which interaction is specific to Rac and crucial for activation of $gp91^{phox}$ [24,25]; Cdc42 neither binds to $p67^{phox}$ nor activates $gp91^{phox}$. Rac binding to $p67^{phox}$ likely induces a conformational change [26], which may render a so-called activation domain in a functional state; the domain resides C-terminal to the TPR domain of $p67^{phox}$ and required for oxidase activation [27,28]. The Rac-activated $p67^{phox}$, in turn, appears to directly interact with $gp91^{phox}$, leading to superoxide production [26,29]. It has also been reported that GTP-bound Rac by itself can make a direct contact with $gp91^{phox}$, contributing to activation of $gp91^{phox}$ [30–32].

According to the above-mentioned model, $p67^{phox}$ is directly involved in activation of $gp91^{phox}$; on the other hand, $p47^{phox}$ functions solely by tethering $p67^{phox}$ to cytochrome b_{558}. Consistent with this, $p47^{phox}$ is dispensable for cell-free activation of the phagocyte oxidase under the conditions where extremely high amounts of $p67^{phox}$ and Rac are present [33–35]. Thus, $p67^{phox}$ is considered to serve as an "activator," whereas $p47^{phox}$ is regarded as an "organizer"; these terms are used for the nomenclature of their

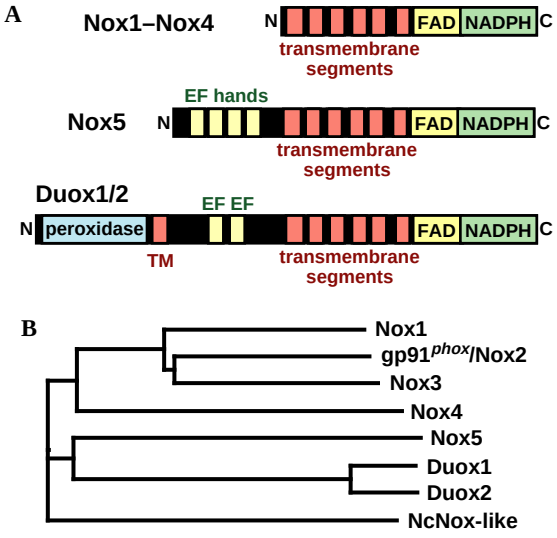

Fig. 4. (A) Schematic structures of human members of the Nox family. (B) Phylogenetic tree of human Nox family oxidases. The $gp91^{phox}$-related domains of the human Nox-family oxidases and a Nox-like oxidase from the fungus *Neurospora crassa* (NcNox-like) were aligned, and the tree was constructed.

homologues (see below). However, it seems still possible that p47^{phox} directly regulates electron transfer for superoxide production, since p47^{phox} (and its homologue Noxo1) can up-regulate the superoxide-producing activity of the gp91^{phox}-related oxidase Nox3 in the absence of p67^{phox} (for details, see the section for Nox3) and a mutant p47^{phox}, defective in the phagocyte oxidase activation, normally recruits p67^{phox} to the membrane [M. Taura and H. Sumimoto, unpublished observation].

In addition to these essential regulators, the adaptor protein p40^{phox} positively regulates activation of gp91^{phox} [36]. p40^{phox} contains an N-terminal PX domain, a single SH3 domain, and an acidic OPCA motif (a.k.a., PC motif)-harboring PB1 domain in the C-terminus (Fig. 3A). An OPCA motif presented on a class of PB1 domains (type I) is used for direct binding to a conserved lysine residue on the first β-strand of another class of PB1 domains (type II), such as the p67^{phox} PB1 domain [37–39]. The PB1-mediated specific heterodimerization allows p40^{phox} to tightly associate with p67^{phox} without affecting the SH3-mediated tail–tail interaction of p67^{phox} with p47^{phox}; as a consequence, p40^{phox}, p67^{phox}, and p47^{phox} form a ternary complex in phagocytes [36]. During cell activation, p40^{phox} facilitates membrane translocation of p67^{phox} and p47^{phox}, leading to an enhanced superoxide production by the NADPH oxidase [36]. The PX domain of p40^{phox} is capable of specifically and strongly binding to phosphatidylinositol 3-phosphate, a lipid enriched in the phagosomal membrane [18,19,40], and thus considered to play a role in recruiting the protein complex correctly to the phagosome.

Regulation of Nox1, the first identified mammalian homologue of gp91^{phox}

It was previously reported that a superoxide-producing enzyme similar to the phagocyte NADPH oxidase exists in vascular smooth muscle cells [41] and gastric pit cells in guinea pigs [42], and the responsible oxidase is currently recognized as Nox1, the first gp91^{phox} homologue to be identified in mammals [43,44]. Nox1 as well as gp91^{phox} contains the N-terminal transmembrane segments and the C-terminal ferredoxin reductase domain, sharing 56% amino acid identity with gp91^{phox} (Fig. 4A). Although Nox1 is abundantly expressed in colon epithelial cells and considered to be involved in host defense at the colon [45,46], this oxidase is also assumed to participate in signal transduction leading to hypertrophy and cell proliferation [43,47].

In contrast to gp91^{phox} expression in limited types of cells such as phagocytes, the mRNA for p22^{phox} is present in various types of cells, including cell lines such as HeLa, COS, and HEK293 cells. Indeed, transfection of these cells with the gp91^{phox} cDNA alone allows reconstitution of functional cytochrome b_{558} [48]. The widespread expression of p22^{phox} may suggest its involvement with other Nox isoforms. Although functional Nox1 can be reconstituted in CHO cells, which scarcely express endogenous p22^{phox} transcripts, Nox1-mediated superoxide production is strongly enhanced by cotransfection of the p22^{phox} cDNA [48]. In addition, p22^{phox} can be complexed with Nox1; expression of Nox1 appears to stabilize p22^{phox} at the protein level, and vice versa [49,50]. Thus, Nox1 likely forms a mutually stabilizing complex with p22^{phox}, as does gp91^{phox}/Nox2. Although it is clear that p22^{phox} is essential for the full activity of Nox1, Nox1 may function in the absence of p22^{phox}; superoxide production by Nox1 expressed in CHO cells is largely but partially dependent on the cotransfection with the p22^{phox} cDNA [48], while the activities of gp91^{phox}/Nox2 and Nox3 absolutely require p22^{phox} expression under the same conditions [48,51].

Mechanism for Nox1 regulation has been uncovered since the discovery of the p47^{phox} homologue Noxo1 (Nox organizer 1) and the p67^{phox} homologue Noxa1 (Nox activator 1) [48,52–54]. Although Nox1 is inactive without an organizer or an activator, it produces a large amount of superoxide in cells coexpressing both Noxo1 and Noxa1, which can be replaced by p47^{phox} and p67^{phox}, respectively, but to a lesser extent [48,52]. Noxo1 has a domain architecture similar to that of p47^{phox}, except the absence of AIR (Fig. 3B), and appears to function via its SH3 domains by binding to the Nox1 partner p22^{phox}. In the presence of Noxo1 and Noxa1, Nox1 produces superoxide without cell stimulants such as phorbol 12-myristate 13-acetate (PMA), a potent in vivo activator of the phagocyte NADPH oxidase, although PMA treatment of cells enhances the superoxide production [48]. The constitutive activity of Nox1 appears to be at least partially due to that Noxo1 can bind to p22^{phox} even in the resting state; the absence of AIR in Noxo1 may render its SH3 domains in a target-accessible state, in contrast with the p47^{phox} SH3 domains [48,60]. Noxa1 can interact with Noxo1 via its C-terminal SH3 domain (Fig. 3B), which interaction may be involved in formation of the active Nox1 complex. Noxa1 as well as p67^{phox} contains a Rac-binding TPR domain in the N-terminal region. Although there is a PB1-like domain in Noxa1, it lacks the conserved lysine residue and thus fails to associate with p40^{phox}.

Rac appears to participate in Nox1 regulation, which is suggested by the following observations. Blockade of the conversion of Rac to the GTP-bound state prevents Nox1-dependent superoxide production in guinea pig gastric mucosal cells stimulated with lipopolysaccharide, which prevention is restored by expression of a constitutively active form of Rac, but not by that of Cdc42 [55]. Both Rac activation and Nox1-dependent ROS production are inhibited in cells depleted of the Rac activator βPix by RNA interference (RNAi) [56]. However, it remains obscure at present whether Rac is directly involved in the active, fully assembled Nox1 complex, as in the phagocyte oxidase complex, or indirectly activates Nox1 via acting in the upstream signaling pathway. The Nox enzymes regulated by Noxa1 or by its homologue p67^{phox} can be directly modulated by Rac. This is because GTP-loaded Rac, but not Cdc42, interacts via the N-terminal TPR domain with Noxa1 as well as with p67^{phox} [48], and the TPR-mediated

association of $p67^{phox}$ with Rac is required for activation of $gp91^{phox}$/Nox2 [24]. Hence, the absolute dependence of Nox1 activity on Noxo1 or $p67^{phox}$ may imply a direct role of Rac. On the other hand, it has been reported that Rac1 can directly bind to the C-terminal region of Nox1 in a GTP-dependent manner [56]; a similar direct binding of GTP-bound Rac to cytochrome b_{558} comprising $gp91^{phox}$/Nox2 and $p22^{phox}$ has been also demonstrated [32]. The precise role of Rac in Nox1 regulation should be clarified in the future studies.

Regulation of Nox3, the oxidase responsible for otoconia formation in mice

The third mammalian NADPH oxidase Nox3, originally described as an enzyme expressed in several fetal tissues such as kidneys and livers [57,58], shows the closest similarity to $gp91^{phox}$ (58% identity) among the Nox-family oxidases (Fig. 4). It is presently known that Nox3 exists also in the inner ear of mice and plays a crucial role in formation of otoconia, tiny mineralized structures that are required for perception of balance and gravity [59]; mice with Nox3 mutations exhibit the *head tilt* (*het*) phenotype because of impaired otoconia formation [59].

As in the case of $gp91^{phox}$/Nox2, Nox3 probably forms a functional complex with $p22^{phox}$. Expression of Nox3 leads to stabilization of $p22^{phox}$ at the protein level and $p22^{phox}$ can be coimmunoprecipitated with Nox3 [51]. In addition, more importantly, coexpression of $p22^{phox}$ is absolutely required for superoxide production by Nox3 in CHO cells, which scarcely express endogenous $p22^{phox}$ mRNA [51].

In contrast to $gp91^{phox}$/Nox2 and Nox1, Nox3 appears to be constitutively active: heterologous expression of Nox3 (and $p22^{phox}$) leads to a spontaneous production of superoxide, which does not require coexpression of an oxidase organizer ($p47^{phox}$ or Noxo1) or an oxidase activator ($p67^{phox}$ or Noxa1), or cell stimulation with agonists such as PMA [51]. In the absence of the activator proteins, the organizers $p47^{phox}$ and Noxo1 are capable of enhancing Nox3 activity via their SH3-dependent interaction with $p22^{phox}$; the effect of $p47^{phox}$, but not that of Noxo1, requires cell stimulation with PMA [51,60]. On the other hand, expression of $p67^{phox}$ by itself leads to enhancement of Nox3-dependent superoxide production in a manner independent of the PRR of $p22^{phox}$. Thus, the organizers and activators, albeit dispensable, can regulate Nox3-dependent superoxide production [51,60,61].

On the other hand, Rac does not seem to be involved in Nox3 regulation [51]. Expression of a dominant negative form of Rac1 does not affect the superoxide-producing activity of Nox3, while it results in an impaired superoxide production by $gp91^{phox}$. A constitutively active form of Rac does not exert any effect on the Nox3-dependent superoxide production under the conditions where the $gp91^{phox}$ activity is largely dependent on the presence of the active Rac. In addition, the $p67^{phox}$-induced enhancement of Nox3 activity does not require the binding of Rac to $p67^{phox}$, which is indispensable to $gp91^{phox}$/Nox2 activation.

It remains unknown whether, in humans, Nox3 is expressed in the inner ears and thus responsible for otoconia formation. Since $p22^{phox}$-deficient CGD patients have no problems in perception of balance and gravity, Nox3 is probably dispensable to otoconia formation in humans. Defective Nox3 activity caused by the absence of $p22^{phox}$ may be compensated by a $p22^{phox}$-independent Nox family oxidase such as Nox5 and Duox in humans (see below). In this context, it seems interesting to consider that Nox5 is not found in the genome of mouse, in which Nox3 is indispensable for otoconia formation. Alternatively, a heretofore unidentified homologue of $p22^{phox}$ might form a functional heterodimer with Nox3 in human ears.

Regulation of Nox4, an oxidase abundantly expressed in the kidney

Compared with Nox1 and Nox3, Nox4 is distantly related to $gp91^{phox}$/Nox2, exhibiting only 39% identity in amino acid sequence (Fig. 4). Nox4 was initially identified as an NAD(P)H oxidase highly expressed in the adult and fetal kidney [62,63]; immunohistochemical study on human renal cortex has detected Nox4 expression in epithelial cells of distal tubules. It is presently known that Nox4 is also expressed in a variety of cells including those in the cardiovasculature, especially endothelial cells [64]. Abundant expression of the $p22^{phox}$ mRNA in the kidney [65] and localization of the $p22^{phox}$ protein in distal tubular cells [66] seem to be indicative of the association of $p22^{phox}$ with Nox4. Indeed, $p22^{phox}$ can be complexed with ectopically expressed Nox4, which seems to lead to its stabilization [49,67]. Although Nox4 produces a small but significant amount of superoxide in a constitutive manner [62,63,67], increase in $p22^{phox}$ expression seems to facilitate the Nox4-dependent ROS production [49,67]. Conversely, RNAi-mediated knockdown of endogenous $p22^{phox}$ transcripts results in a reduced Nox4 activity [67]. These findings suggest that Nox4 forms a complex with $p22^{phox}$ to function as a superoxide-producing oxidase.

A little is known whether the oxidase organizers and activators play a role in Nox4 regulation. Neither coexpression of $p47^{phox}$ and $p67^{phox}$ nor that of Noxo1 and Noxa1 has any effect on the Nox4-dependent ROS production [49,67], suggesting that these regulatory proteins are not involved. In addition, Rac does not appear to modulate the constitutive Nox4 activity. It has recently been shown, using cells ectopically expressing Nox4, that Rac1 silencing via RNAi does not affect ROS production by Nox4, and that neither a constitutively active Rac1 nor a dominant negative Rac1 exerts an effect on the Nox4 activity [67]. By contrast, it has been reported that treatment of renal mesangial cells with angiotensin II induces Nox4-mediated superoxide generation in a Rac-dependent manner [68]. In this system, Rac possibly acts in a direct manner, since cell-free activation with arachidonic acid is blocked in

homogenates prepared from Rac1(T17N)-expressing mesangial cells. However, the possibility that gp91^{phox}/Nox2, an oxidase known to be also present in mesangial cells, becomes activated in the cell-free system is not completely excluded. Further studies are required for clarifying mechanism for Nox4 regulation at the molecular level.

Regulation of Nox5, the oxidase containing four EF hands

Human Nox5 is abundantly expressed in T- and B-lymphocytes of spleen and lymph nodes, and in the sperm precursors of testis [69]; however, its biological role is presently unknown. Intriguingly, no orthologue for Nox5 is found in the mouse and rat genomes. Nox5 builds on the basic structure of gp91^{phox}, adding an N-terminal extension that contains four Ca^{2+}-binding sites: three canonical and one non-canonical EF-hands (Fig. 4). The gp91^{phox}-like domain of Nox5 exhibits only 22–27% amino acid identity to those of the other four Nox enzymes. Nox5 is rather related to Duox enzymes (see the following section); EF-hand motifs are also present in Duox but not in Nox1 through Nox4 (Fig. 4).

As expected by the existence of EF-hands, Nox5 is activated by Ca^{2+} to produce superoxide: cells ectopically expressing Nox5 produce superoxide in response to the Ca^{2+}-ionophore ionomycin [69]; cell-free superoxide production by Nox5-containing membrane fractions is dependent on the presence of Ca^{2+} [70]. It has been suggested that the Ca^{2+}-binding domain of the Nox5 N-terminus may function as a calmodulin-like activator module: the binding of Ca^{2+} causes a conformational change, which leads to intramolecular interaction of the N-terminal calmodulin-like module with the C-terminal gp91^{phox}-like domain, culminating in activation of the NADPH oxidase [70]. Nox5 does not seem to form a functional complex with p22^{phox}, since knock down of p22^{phox} via RNAi does not affect the Nox5-dependent superoxide production under the conditions where it abrogates the activity of Nox1–4 [50].

Regulation of the dual oxidases Duox1 and Duox2

Besides a C-terminal NADPH oxidase domain, Duox enzymes feature an N-terminal peroxidase-like ectodomain that is separated from two EF hands by an additional transmembrane segment (Fig. 4), and thus called "dual" oxidases [5]. However, it is not clear whether the N-terminal domains of Duox have an appreciable peroxidase activity. Although the ectodomains are thought to belong to the MPO (myelopreoxidase)-related preoxidase family because of considerable homology in the entire regions, they lack both or one of the His residues that are involved in the heme binding and thus conserved among the other members including thyroid peroxidase (TPO), lactoperoxidase, and ovoperoxidase [71].

The genes highly homologous to the gp91^{phox}-encoding one such as those for Nox1–4 (Fig. 4) are, to date, known to exist only in the phylum Chordata: the homologues are found not only in vertebrates but also in ascidians [72]. On the other hand, Duox-type enzymes are present in a wide variety of animals including *Caenorhabditis elegans* (the phylum Nematoda), *Drosophila melanogaster* (the phylum Arthropoda), and sea urchins (the phylum Echinodermata). The Duox1 of *C. elegans* is involved in cross-linking of tyrosine residues of extracellular matrix proteins, thereby facilitating cuticle formation [73]. The rapid increase in oxygen consumption (respiratory burst), in conjunction with H_2O_2 generation, was known to occur during fertilization of sea urchins. A recent study [74] has demonstrated that a sea urchin Duox homologue, Udx1, is responsible for generating the H_2O_2 necessary for formation of the fertilization envelope as the physical block to polyspermy.

Biosynthesis of thyroid hormones in mammals requires H_2O_2, with which TPO catalyzes conjugation of iodide ions to tyrosine residues on thyroglobulin in the thyroid follicles, an essential step for synthesizing an active hormone. The H_2O_2 is generated in an NADPH-dependent manner by Duox expressed at the apical surface of thyrocytes (a.k.a. Thox) [75,76]. Mutations in Duox2, one of the two human Duox homologues, are associated with a loss of thyroid hormone synthesis and can lead to permanent and severe congenital hypothyroidism [77]. Although Duox1 is also expressed in thyrocytes, its significance remains unclear at present.

The Duox enzymes are expected to produce superoxide as an initial product, on the basis of homology of their gp91^{phox}-like oxidase domains with those of the superoxide-generating Nox oxidases. However, mature Duox enzymes are known to release H_2O_2 without forming a detectable amount of superoxide [78]. It has recently been reported that superoxide can be detected when an immature partially glycosylated Duox is used [79]. In mature Duox, superoxide produced by the gp91^{phox}-like domain may be rapidly converted to H_2O_2 via intramolecular dismutation.

It is well established that mammalian thyroid oxidase and sea urchin NADPH oxidase are reversibly activated with Ca^{2+}. A recent study has shown that a partially glycosylated form of Duox2, located in the endoplasmic reticulum, generates ROS in a Ca^{2+}-dependent manner [79]. Regulation of Duox by Ca^{2+} is considered to occur via its paired EF-hand domain. It is known that limited proteolysis with α-chymotrypsin renders thyroid NADPH oxidase in a fully and irreversibly active state independent of Ca^{2+} [80]. The Ca^{2+}-binding domain of Duox may serve as an autoinhibitory domain rather than as an activation domain, as proposed for that of Nox5 [70]; the inhibition may be released reversibly by physiological Ca^{2+}-induced conformational change and irreversibly by proteolytic removal of the autoinhibitory domain [79]. On the other hand, a Ca^{2+}-independent pathway may also occur for Duox activation, since ROS production in thyrocytes can be induced in response to PMA [81], an agent that is capable of directly activating PKC but not expected to elevate

cytoplasmic concentration of Ca^{2+}. A PKC-dependent pathway is also suggested to be involved in H_2O_2 generation at fertilization in sea urchin [74].

It has recently been reported that $p22^{phox}$ can be co-immunoprecipitated with Duox using anti-Duox antibodies in primary cultured human thyrocytes [82]. However, it seems unlikely that Duox forms a functional complex with $p22^{phox}$. Although Duox2 is essential for thyroid hormone biosynthesis as described above, hypothyroidism is not associated with the $p22^{phox}$-deficient type of CGD, indicating that Duox2 activity does not require $p22^{phox}$. Consistent with this, coexpression of $p22^{phox}$ has no effect on the reconstituted H_2O_2-generating activity of Duox2, albeit in an immature form, expressed in CHO and HEK293 cells [79]. In addition, a homologue of $p22^{phox}$ exists in the genomes of vertebrates and ascidians [72] but not in those of *C. elegans* and *Drosophila*. The latter two genomes also contain Duox genes, indicating that Duox basically functions without $p22^{phox}$.

Duox oxidases likely function in the absence of $p47^{phox}$ and $p67^{phox}$, since they cannot be detected in the thyroid. In agreement with this, hypothyroidism does not occur in CGD patients lacking $p47^{phox}$ or $p67^{phox}$. Although it has not been intensively tested whether Noxo1 or Noxa1 regulates Duox activity, Duox may also act without them; neither homologues of $p47^{phox}$ nor those of $p67^{phox}$ are found in the *C. elegans* and *Drosophila* genomes.

Involvement of the small GTPase Rac in Duox activity also seems unlikely. The Ca^{2+}-ionophore ionomycin, an agonist for Duox, is incapable of inducing conversion of Rac to the GTP-bound active form in dog thyroid cells [81]. In addition, ionomycin- or carbamylcholine-stimulated H_2O_2 generation in canine thyrocytes is not affected by pretreatment with the *Clostridium difficile* toxin B, an agent that inhibits Rac proteins and the activation of $gp91^{phox}$ in phagocytes [81].

Cooperation between Nox oxidases and MPO-like peroxidases

Nox family oxidases often function in cooperation with MPO-like peroxidases. In the phagosome, superoxide released by the phagocyte oxidase $gp91^{phox}$ is dismuted into H_2O_2, which is further converted to HOCl, a powerful fungicidal agent, by MPO—a peroxidase that is specifically expressed in granules of neutrophils and monocytes, and released to the intraphagosomal space [1,83]. MPO thus contributes to host defense in a manner dependent on the phagocyte NAPDH oxidase activity [84]. In thyroid hormone synthesis, Duox2 collaborates with the thyroid-specific peroxidase TPO. Duox oxidases are also present in salivary glands and mucosal epithelium, and appear to function together with lactoperoxidase to play important roles in mucosal surface host defense [85]. In the sea urchin egg, Udx1 supplies H_2O_2, with which ovoperoxidase catalyzes cross-linking of fertilization envelope proteins, thereby completing a competent block to polyspermy [74]. Daiyasu and Toh [71] have shown that a region N-terminal to the catalytic domain of MPO (Region I), responsible for dimerization, is conserved in members of the MPO-related peroxidase family, including the Duox ectodomains. It is tempting to postulate that Duox assembles via Region I to function effectively or Duox directly interacts via Region I with its partner peroxidase. Indeed, Duox2 is known to form a complex with TPO in human thyocytes [82].

Another look

Among Nox-family oxidases, members of the Duox subfamily are found in a variety of animals. On the other hand, the existence of the closest Nox members to $gp91^{phox}$ (Nox1–4) is confined to the phylum Chordata. These Nox enzymes form a functional complex with $p22^{phox}$, in contrast to Nox5 and Duox1/2. The requirement for $p22^{phox}$ appears to arise from its functional role, especially regulation by the organizers and activators ($p47^{phox}$, $p67^{phox}$, and their homologues), since the $p22^{phox}$ gene and the genes for these regulatory proteins emerge simultaneously, i.e., at the emergence of the chordate; the genes are not revealed by the analysis of available genomes from other animal phyla, plants, or fungi. The most closely related oxidases Nox1–3 are controlled in a $p22^{phox}$-dependent manner, which includes direct binding of $p47^{phox}$ or Noxo1 to the C-terminal PRR of $p22^{phox}$ [48,51,60]. For appropriate regulation of Nox activity, the $p22^{phox}$ subunit should associate tightly with Nox catalytic subunits on the membrane. It seems possible that, during the course of chordate evolution, mutations in Nox oxidases such as $gp91^{phox}$/Nox2 have accumulated so that catalytic subunits cannot exist stably without $p22^{phox}$ anymore, even though $p22^{phox}$ may be initially created for increase in a diversity of regulatory mechanisms. In this context, it should be noted that the Nox4 protein, less close to $gp91^{phox}$, is stable even in the absence of $p22^{phox}$, albeit Nox4-dependent ROS production is regulated by $p22^{phox}$ [50]. The $p22^{phox}$-independent members Nox5 and Duox1/2, as well as plant NAD(P)H oxidases, instead harbor EF-hands at the N-terminus, via which they are activated by temporal elevation in cytoplasmic Ca^{2+} concentration. Such a direct regulation by Ca^{2+} does not occur in other Nox oxidases lacking the Ca^{2+}-binding motif. On the other hand, PMA, a potent agonist for PKC, is capable of activating not only Nox1–3 but also Duox [48,51,81]. The PKC-dependent Nox regulation involves phosphorylation-induced conformational change of $p47^{phox}$, whereas $p47^{phox}$ does not participate in Duox modulation.

Among the Nox-family oxidases in animals, the direct involvement of Rac appears to be confined to a few members, i.e., $gp91^{phox}$/Nox2 and probably Nox1. This may be unexpected, given that Rac is an essential component in the active complex of $gp91^{phox}$. Although spatial regulations of Nox are not well understood, the direct involvement of Rac likely plays a crucial role in localizing the active phagocyte oxidase complex exclusively to the site where Rac

activation occurs, e.g., the phagosomal membrane. If Rac controls other oxidases as an indirect upstream regulator, the regulation would occur in less restricted areas. Rac also modulates NAD(P)H oxidases in plants [86] and possibly in fungi [87], although it is presently unknown whether the effects are direct or indirect.

Recent studies have thus revealed that Nox is controlled by a diversity of post-translational regulatory mechanisms, which allows regulated cellular (and subcellular) ROS levels for a variety of biological events. The precise mechanisms for the temporal and spatial regulations of Nox-mediated ROS production remain an area for further investigation.

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