ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 10, 2009

DOI: 10.1089/ars.2009.2637

FORUM REVIEW ARTICLE

Targeting and Regulation of Reactive Oxygen Species Generation by Nox Family NADPH Oxidases

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Abstract

Nox family NADPH oxidases serve a variety of functions requiring reactive oxygen species (ROS) generation, including antimicrobial defense, biosynthetic processes, oxygen sensing, and redox-based cellular signaling. We explored targeting, assembly, and activation of several Nox family oxidases, since ROS production appears to be regulated both spatially and temporally. Nox1 and Nox3 are similar to the phagocytic (Nox2-based) oxidase, functioning as multicomponent superoxide-generating enzymes. Factors regulating their activities include cytosolic activator and organizer proteins and GTP-Rac. Their regulation varies, with the following rank order: Nox2 > Nox1 > Nox3. Determinants of subcellular targeting include: (a) formation of Nox-p22^{phox} heterodimeric complexes allowing plasma membrane translocation, (b) phospholipids-binding specificities of PX domain-containing organizer proteins (p47^{phox} or Nox organizer 1 (Noxo1 and p40^{phox}), and (c) variably splicing of Noxo1 PX domains directing them to nuclear or plasma membranes. Dual oxidases (Duox1 and Duox2) are targeted by different mechanisms. Plasma membrane targeting results in H_2O_2 release, not superoxide, to support extracellular peroxidases. Human Duox1 and Duox2 have no demonstrable peroxidase activity, despite their extensive homology with heme peroxidases. The dual oxidases were reconstituted by Duox activator 2 (Duoxa2) or two Duoxa1 variants, which dictate maturation, subcellular localization, and the type of ROS generated by forming stable complexes with Duox. *Antioxid Redox Signal.* 11, 2607–2619.

Introduction

Reactive oxygen species (ROS) have long been considered in a pathological context as culprits that inflict tissue damage under conditions of chronic or acute inflammatory disease. Their production can be incidental to enzymatic reactions involving oxygen intermediates or, in the case of phagocytic cells, they are produced deliberately by NADPH oxidase activation to kill microbial pathogens during phagocytosis. Within the last decade, there has been a newfound appreciation of deliberate ROS production in a variety of essential biological processes. These include developmental and differentiation processes, extracellular matrix cross-linking, hormone biosynthesis, cellular senescence, apoptosis, responses to oxygenation (oxygen sensing), and cellular signaling responses to growth factors, hormones, and cytokines. The recognition of these new roles for ROS has occurred in parallel with the discovery of an entire family of ROS-generating NADPH oxidases related to the phagocytic system, now known as Nox enzymes. Among the seven known mammalian Nox family NADPH oxidases (Nox1–Nox5, Duox1, and Duox2), several are known to serve essential roles based on the effects of spontaneous or disease-related mutations and targeted gene disruption. Defects in the phagocytic (Nox2-based) oxidase have been known for decades, resulting in chronic granulomatous disease (CGD), which is characterized by enhanced susceptibility to microbial infection and dysregulated inflammation. Recent Nox1 knockout and overexpression studies reveal a role of Nox1 in angiotensin II-based regulation of vascular tone (20, 25, 38, 68). A Nox3-based oxidase is required for development of otoconial structures in the inner ear; mutations in this system cause deficits in balance and gravity perception in the inner ear (60, 78, 80). The effects of Duox2 system mutations reveal its essential role in thyroid hormone biosynthesis in man and in the mouse (51, 74, 111). Recent surveys of the evolutionary origins of Nox family suggest that deliberate ROS production by these oxidases occurs in all multicellular organisms in both plant and animal kingdoms (9, 57).

In all of these Nox-related functions, it appears that ROS production is subject to tight regulation, both spatially and temporally, in order to avoid collateral damage to oxidant-sensitive host cell components. The respiratory burst oxidase mounted by phagocytic cells normally generates

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potent oxidants within the confines of the phagosome to kill ingested microbes (103). Recent studies on the composition, biosynthesis, subcellular targeting, and function of the novel Nox family oxidases suggest a variety of mechanisms by which oxidative tissue damage is minimized by controlling the amount, duration, and specific sites of ROS generation. Redox-based intracellular and extracellular signaling can be limited to particular membrane domains or compartments, designed for localized signaling at sites such as at the leading edge of migrating cells, in endosomes, or in receptor signaling complexes (105). Nox4 was proposed to function in some cells as an intracellular signaling oxidase, consistent with its detection in nuclear or perinuclear regions (3, 39, 61, 67). In cases involving Duox isozymes in polarized epithelium, high hydrogen peroxide (H_2O_2) production is limited to the apical plasma membrane for biosynthetic purposes or other reactions supported by extracellular peroxidases (e.g., thyroperoxidase (TPO), lactoperoxidase (LPO), ovoperoxidase) (26). Thus, a complete understanding of their function as deliberate ROS generators requires an appreciation of the factors that regulate their activity and target the active enzymes to particular subcellular compartments, as well as the types of ROS they generate at these sites. This review provides an update on the regulation and function of several Nox family NADPH oxidases, emphasizing our recent findings in reconstituted oxidase systems, where we have explored factors affecting subcellular localization of oxidase components and the type of ROS produced.

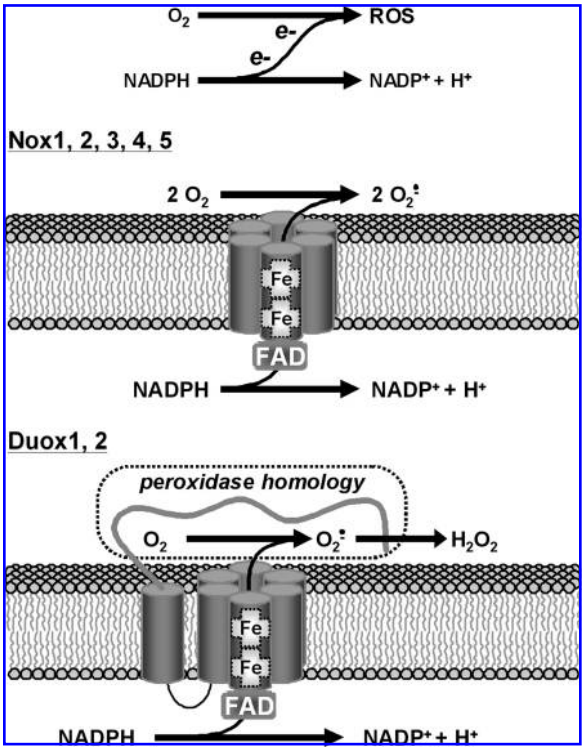

FIG. 1. Generation of reactive oxygen species (ROS) by Nox family NADPH oxidases. All are electrogenic enzymes that accept electrons from cytosolic NADPH, transport them through FAD and membrane-imbedded hemes, and donate single electrons to molecular oxygen, thereby producing superoxide anion. In the case of the dual oxidases (Duox1 and Duox2), a superoxide intermediate is not readily detected and the N-terminal peroxidase-like domain appears to affect its conversion into H_2O_2 by undefined mechanisms. The most structurally conservation features of these enzymes include regions of the C-terminal reductase domain that bind NADPH and FAD and the membrane-spanning helical segments thought to bind heme.

The ROS-Generating Catalytic Mechanism of Nox Family Oxidases

All Nox family NADPH oxidases are transmembrane electron carriers that use NADPH as an electron source and molecular oxygen as an acceptor (Fig. 1). They function as electrogenic enzymes that pass electrons from the cytoplasm through a membrane into some extracytoplasmic space, either the cell exterior or intracellular compartments such as phagosomes, endosomes, the endoplasmic reticulum, or perinuclear compartment. The core catalytic Nox components all contain C-terminal reductase domains, with conserved NADPH and FAD-binding sequences. These domains are anchored to a common heme-containing domain composed of six membrane-imbedded alpha-helical segments. In the case of the Nox enzymes, helix 3 and 5 each contain two invariant histidine residues located near the membrane interfaces that coordinate two heme molecules. The Dual oxidases (Duox1 and Duox2) share these features, but have an additional N-terminal extension with another transmembrane segment preceded by a large extracellular domain showing sequence similarities to several heme peroxidases.

According to the proposed ROS-generating mechanism deduced from the best characterized Nox2-based or *phox* system of phagocytic cells, one NADPH molecule yields two superoxide ions in a stepwise transmembrane transfer of two single electrons to two molecules of oxygen (21). The effective range of superoxide released into phagosomes is limited, given its instability as a charged free radical and the high levels of superoxide dismutase in this compartment, although recent work suggests the ClC-3 anion channel transports superoxide through membranes (73, 76). Spontaneous or catalytic dismutation of superoxide into a more stable and membrane-permeable product, H_2O_2, permits its detection even outside of the cell. Most reconstituted cells expressing nonphagocytic Nox enzymes (*i.e.*, Nox1, Nox3, and Nox5) release extracellular superoxide, suggesting these oxidases reach the plasma membrane and that the same Nox2-based ROS-generating mechanism also applies to these oxidases (7, 56, 100). In several studies, H_2O_2 is the predominant ROS detected in Nox4-reconstituted cells, consistent with its accumulation within intracellular sites (3, 67). Extracellular superoxide can be detected in Nox4-reconstituted cells by the sensitive Diogenes luminescence assay system (39) or by nitro-blue tetrazolium, a membrane-permeable reagent that reaches intracellular compartments (89). Other studies claim Nox4 is unique in its ability to produce H_2O_2 more directly, although the mechanisms involved are not clear (48, 108).

Dual oxidases exhibit an important feature distinct from the other Nox isozymes, in that they produce only H_2O_2, not superoxide. The Duox ancestor, the mysterious "thyroid H_2O_2 generator", was known to be an NADPH oxidase producing only H_2O_2 in a calcium-dependent manner (24, 27, 77, 91, 107). Duox1 and Duox2 cloning from thyroid tissues confirmed the

Nox-like features of these enzymes and revealed an additional transmembrane helix preceded by a long extracellular N-terminal region that is ~20% homologous with hemoperoxidases (23, 28). This domain most likely confers the unique property of H_2O_2 production. Recent studies on Duox1 and Duox2 reconstituted in plasma membranes by co-expression of their respective maturation factors confirm these oxidases produce H_2O_2 from the cell surface, and no detectable superoxide (44, 72).

The Nox enzymes can be classified into three distinct functional groups (Fig. 2) related to the overall composition of the active enzymes: (a) Nox1, Nox2, and Nox3 function as multicomponent complexes involving cytosolic regulator proteins, (b) Nox4 exhibits high constitutive activity and is not dependent on any of the regulators that support Nox1, Nox2, or Nox3, and (c) Nox5, Duox1, and Duox2 are regulated by increased intracellular calcium levels due to the presence of calcium-binding EF-hands (8, 41, 63, 93). Direct interactions between the calcium-bound EF-hands and the C-terminal reductase domains were shown to activate Nox5 (7) and many presume the Duox isozymes are activated through a similar mechanism.

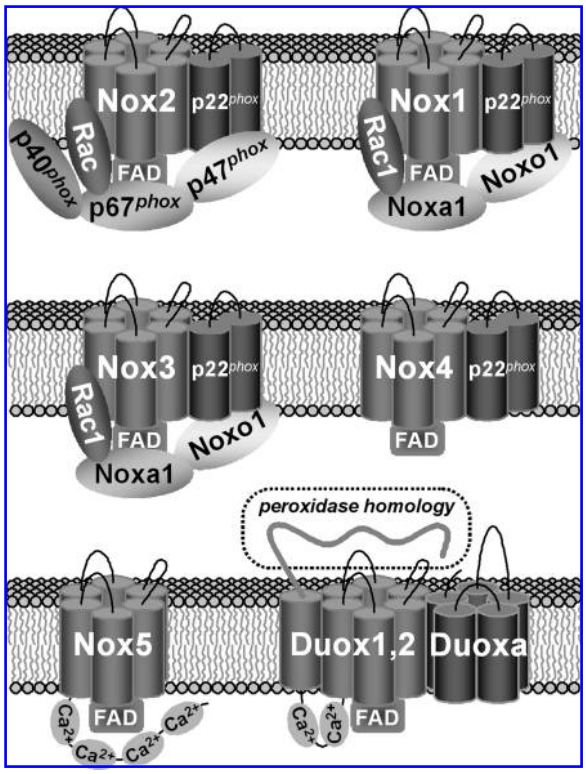

FIG. 2. Molecular components of active Nox family NADPH oxidase complexes. Nox1, Nox2, and Nox3 function as regulated enzymes involving cytosolic adaptor proteins or "Nox organizers" ($p47^{phox}$ or Noxo1 and $p40^{phox}$) and "Nox activators" ($p67^{phox}$ or Noxa1) that bind GTP-Rac and affect the flow of electrons. The $p22^{phox}$ component forms a stable heterodimeric complex with Nox core components (Nox1–4), required for post-translation processing or maturation into active oxidases. In Nox1–Nox3 systems, $p22^{phox}$ also promotes plasma membrane targeting of the oxidases and provides a docking site for Nox organizers. Nox5 and Duox are calcium-responsive oxidases that contain calcium-binding EF-hands. The Duox activators (Duoxa) are maturation factors functionally similar to $p22^{phox}$ recently shown to form stable complexes with Duox on the plasma membrane (72).

In most cases (excluding Nox5), the functional unit of these oxdases is considered a heterodimeric complex formed between the core oxidase flavocytochrome component and another membrane-imbedded chain, either $p22^{phox}$ or Duox activator (Duoxa) components, that serves as a "maturation factor" involved in their complete biosynthesis. The importance of Nox2 ($gp91^{phox}$) and $p22^{phox}$ co-expression has been evident in CGD patients, where genetic defects affecting production of either component results in significantly reduced levels of the other chain, indicating that stabilization of the flavoenzyme involves formation of the heterodimeric complex (82, 87). Studies on the biosynthesis of Nox2 indicated that formation of this complex is a requisite for several post-translational modifications (heme incorporation and glycosylation), as well as for protein translocation beyond the endoplasmic reticulum (ER) (113). Subsequent studies suggested Nox1, Nox3, and Nox4 all form complexes with $p22^{phox}$ (3, 67, 98, 100). We showed that heterologous overexpression of either Nox1 or Nox3 results in cellular redistribution of $p22^{phox}$ from an ER-like pattern to accumulation along the plasma membrane, which is accompanied by enhanced $p22^{phox}$ detection by immunofluorescence and Western blotting (Fig. 3). The findings are consistent with release of superoxide by cells reconstituted with these oxidases (100) and plasma membrane co-localization of $p22^{phox}$ with Nox1, Nox3, or Nox4 in several tissues where the native oxidases are most abundant (58, 78). In addition to affecting oxidase targeting and stabilization, $p22^{phox}$ serves yet another function in the Nox1-, Nox2-, and Nox3-based systems in providing a docking site for their cytosolic regulators (see below). Mutagenesis experiments suggest this $p22^{phox}$ feature is not conserved in the Nox4-based enzyme, consistent with its ability to function independent of known oxidase cytosolic regulators (58, 108). Further studies are needed to clarify whether $p22^{phox}$ is essential for the Nox4-based enzyme or some other factor allows Nox4 to escape the ER.

The Duox isozymes also rely on maturation factors (a.k.a. Duox activators; Duoxa1, or Duoxa2) in order to reconstitute active Duox on the plasma membrane (44). Duoxa2 was initially proposed to act as an ER-resident protein required to export Duox beyond this compartment, however our recent work indicates the Duox maturation factors act in a similar fashion as the $p22^{phox}$ counterpart by forming stable complexes with the active oxidases on the plasma membrane (72).

Regulation of Multicomponent Oxidases: Nox1–Nox3

The activities of Nox1, Nox2, and Nox3 are all subject to the influence of cytosolic regulators. These regulatory mechanisms involve three types of factors: a) the small GTPase Rac bound to GTP, b) its GTP-dependent target proteins, referred to as Nox activators ($p67^{phox}$ or Noxa1), and c) the organizer or adaptor proteins ($p47^{phox}$ or Noxo1, and $p40^{phox}$) responsible for bridging interactions of the Nox activators with the flavocytochrome components. The extent to which these oxidases are regulated varies, with the following rank order: Nox2 > Nox1 > Nox3, where Nox2 is completely inactive in

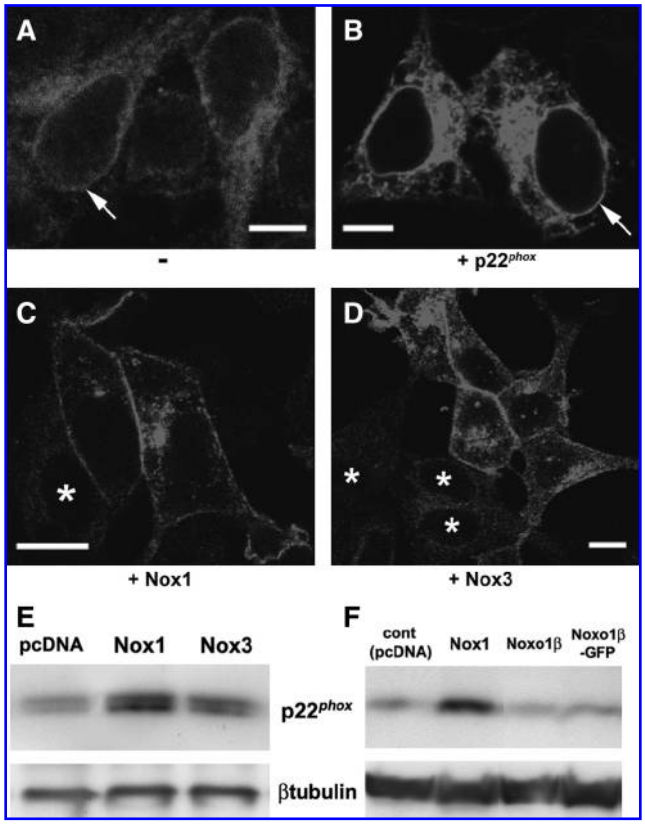

FIG. 3. Nox 1 and Nox 3 expression enables plasma membrane targeting and stabilization of p22^{phox}. Immunofluorescence imaging of endogenous (**A**) and transfected (**B**) p22^{phox} in HEK293 cells, showing reticular (ER) and nuclear membrane (*arrows*) staining patterns. (**C**) Transfection of Nox1 results in a redistribution of endogenous p22^{phox} to the plasma membrane. (**D**) A similar redistribution of p22^{phox} occurs in Nox3-transfected HEK293 cells. The untransfected cells (*) show primarily cytosolic staining patterns and overall weaker staining. Western blot analysis shows increased endogenous p22^{phox} levels in Nox1- or Nox3- transfected cells (**E**), but not in mock or Noxo1β-transfected HEK293 cells (**F**). (Modified with permission from Fig. 6 of (100) and Fig. 6 of Ref. (102)).

the absence of cell stimulation, and Nox3 exhibits constitutive activity even in the absence these regulators. The importance of Rac2, p47^{phox}, and p67^{phox} as essential Nox2 system components is evident in autosomal recessive CGD patients whose genetic lesions affect these proteins (65, 79, 83). The maintenance of Nox2 cytosolic regulators in inactive conformations in large part accounts for the latency of this enzyme in unstimulated cells.

After the identification of p47^{phox} and p67^{phox} homologs (41), three groups explored the roles of these proteins (Noxo1 and Noxa1) in supporting Nox1 activity (7, 40, 96). The Nox organizers (p47^{phox} and Noxo1) share common structures and function properties of binding to phospholipids through their PX (phox) domains, to proline-rich (PR) sequences of p22^{phox} through the two SH3 domains, and to SH3 domains of the Nox activators through PR motifs near their carboxyl termini (Fig. 4). The PX domains of p47^{phox} and Noxo1 exhibit different phospholipid-binding specificities: p47^{phox} binds to phosphatidic acid (PA) and PI(3,4)P_2 (54), whereas Noxo1 binds to PA and PI(4)P, PI(5)P, and PI(3,5)P_2 (18, 95, 102). Noxo1 lacks sequence homologous to the C-terminal "autoinhibitory (AIR)" domain of p47^{phox}, encompassing a polybasic region with several serines that are phosphorylated during assembly and activation of the Nox2 complex (35). Both the absence of an AIR in Noxo1 and its phospholipid-binding specificity could explain its binding to the plasma membrane in the absence of cell stimulation (19). The Nox activators (p67^{phox} and Noxa1) share homologous tetratricopeptide (TRP) scaffolding structures that present Rac-binding sequences, conserved activation domains (AD), and PB1 (Phox and Bem1) domains (Fig. 4). Noxa1 lacks a central SH3 domain and its PB1 domain can not bind p40^{phox} (96), although the function of this SH3 domain of p67^{phox} remains unknown. The p40^{phox} component is a second adaptor protein that bridges contacts between membrane lipids (PI(3)P) through its PX domain and p67^{phox} through PB1 domain heterodimerization (Fig. 4). Noxa1 shows a broader tissue-distribution pattern than Noxo1 (40, 96), whereas the expression of Noxo1 is upregulated by inflammatory agents, LPS and TNF-α (55, 62).

In unstimulated phagocytes, the phagocyte oxidase (Nox2) is dissociated and inactive: the membrane-penetrated flavocytochrome b_{558} (gp91^{phox}-p22^{phox}) is stored on intracellular granules (13), the other phox proteins (p47^{phox}, p67^{phox}, and p40^{phox}) associate in a separate ternary cytosolic complex (64) in a dephosphorylated state (12, 85, 88), and Rac is maintained in a GDP-bound cytoplasmic complex dimerized with Rho-GDI (1). During phagocyte activation, intracellular granules containing flavocytochrome b_{558} fuse to newly forming phagosomes, and the ternary cytoplasmic phox complex and Rac bind to these membranes by independent mechanisms (49). p47^{phox} is phosphorylated (2, 35, 103), thereby inducing conformational changes (unmasking) that promote the interaction of the ternary phox cytoplasmic complex with the flavocytochrome b_{558} through interactions between its SH3 domains and p22^{phox} and between its PX domain and membrane phospholipids (45, 54, 66, 94). The "SuperSH3" model specifying AIR interactions responsible for masking of the tandem SH3 domains has been well recognized (45), however, details about precise intramolecular contacts responsible for masking the PX domain of p47^{phox} remain unclear. We reported that residues 341–360 enhance autoinhibitory intramolecular interactions between the PX domain and a structure encompassing the tandem SH3s and the AIR (101) (Figs. 4 and 5). In addition, involvement of the linker region between the PX domain and N-terminal SH3 domain in masking was reported recently (90). Finally, Rac translocates to phagosomes (49, 99) and binds to its effector p67^{phox} in a GTP-dependent manner in a critical regulatory point in Nox2 activation (112), resulting in generation of superoxide anion by the transfer of electrons from cytoplasmic NADPH to molecular oxygen.

Recent studies using p40^{phox} knockout mice and FcγIIA receptor-reconstituted COS^{phox} cells indicate that p40^{phox} is also an essential positive regulator of the Nox2 system (33, 92). PI(3)P binding to the PX domain of p40^{phox} plays critical roles in activation of Nox2 system (16, 32, 34, 52), particularly during FcγR-mediated phagocytosis (106). In contrast, a PX domain-independent, but PI(3)P-dependent, regulatory mechanism occurs in CD18-dependent activation of Nox2

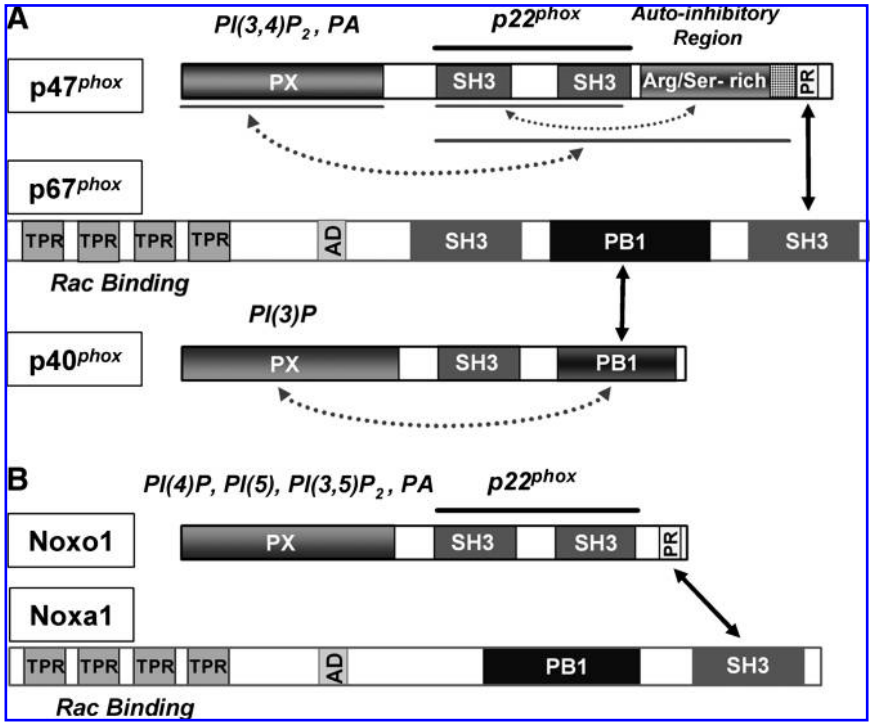

FIG. 4. Schematic representation of multiple modular domains in Nox organizer and activator proteins in phagocytic (A) and non-phagocytic systems (B). The Nox organizers ($p47^{phox}$ and Noxo1) share common structures and functional properties of binding to phosphatidic acid (PA) and phosphoinositide lipids through PX domains, to $p22^{phox}$ through two SH3 domains, and to SH3 domains of Nox activators through the proline-rich (PR) motifs at their carboxyl termini. The Nox activators ($p67^{phox}$ and Noxa1) also share homologous tetratricopeptide repeat (TRP) scaffolds that present Rac binding sequences and activation domains (AD), and PB1 (Phox and Bem1) domain. The $p40^{phox}$ component is a secondary adaptor that bridges contacts between membrane (PI(3)P) and $p67^{phox}$, using PX (phox) domain and PB1 domain heterodimerization, respectively. Autoinhibitory intramolecular interactions that maintain closed conformations in $p47^{phox}$ and $p40^{phox}$ are depicted with *dashed lines*. *Striped box* in $p47^{phox}$ between AIR and PR motif represents residues 341–360, which enhance the autoinhibitory interaction between the PX domain and the structure constructed by the tandem SH3s+ the AIR (101).

system, and a PI(3)P-independent function of $p40^{phox}$ was reported recently (5, 10, 97). We demonstrated that a PX–PB1 domain intramolecular interaction within $p40^{phox}$ renders $p40^{phox}$ inaccessible to interact with PI(3)P in its resting state, and proposed that during Nox2 activation the PX–PB1 interaction can be disrupted, enabling $p40^{phox}$ to bind to PI(3)P-enriched phagosomes (104). A structural analysis of the PX-PB1 autoinhibitory contacts within $p40^{phox}$ was reported (50). Subsequently, we demonstrated that $p40^{phox}$ prolongs the retention of the $p47^{phox}$-$p67^{phox}$-$p40^{phox}$ complex on closed phagosomes and proposed a switching of phagosomal membrane adaptor functions from $p47^{phox}$ to $p40^{phox}$: $p47^{phox}$ functions as the initial adaptor protein bringing the ternary complex to phagosomes, whereas $p40^{phox}$ functions as a late stage adaptor protein to retain the complex on phagosomes during the FcγR-mediated oxidative burst (101) (Fig. 5). However, there are still controversies and unanswered questions about this assembly process, such as the mechanisms promoting the $p40^{phox}$ conformational changes.

Nox1 dimerized with $p22^{phox}$ is activated by related cytosolic regulators, Noxo1, Noxoa1, and Rac1 by mechanisms that are less stringent but very similar to the Nox2 system. Interestingly, the murine Nox1 system exhibits high constitutive activity (6), whereas the human Nox1 system has a PMA-stimulated component showing 1.5- to 3.0-folds increases in activity (40, 100, 102). A negative regulatory mechanism by protein kinase A-mediated phosphorylation of Noxa1 Ser172 and Ser461 was described, which are not conserved in murine Noxa1 (59). Nox3 dimerized with $p22^{phox}$ show species specific differences: the reconstituted murine Nox3 system requires both Noxa1 and Noxo1 for its maximal activation (60), while the human Nox3 system shows maximal activity with Noxo1 alone (98, 100). In contrast to the well-recognized role of Rac in the Nox2 system, demonstration of Rac involvement in the Nox1 and Nox3 systems was less convincing using the same functional criteria. Constitutively active forms of Rac (RacQ61L or RacG12V) or dominant-negative Rac (RacT17N) did not affect these reconstituted cell systems, because of the lower demand for Rac by these oxidases. We demonstrated Rac1 involvement in Nox1 and Nox3 system based on three experiments designed in reconstituted cell models (100): (a) using a membrane-targeted form of Noxa1 (Noxa1pp) that supports Nox1 or Nox3 independent of Noxo1; this Noxa1 mutant was adapted with the C-terminal 10 amino acids of Rac1 (KKRKRKCLLL). This activity was dependent on an interaction with active Rac1; (b) using the Noxa1 mutant (Noxa1R103E) defective in Rac1 binding or the specific Rac1 mutant (Rac1G30S) defective in Noxa1 binding (mutations directed at effector contacts sites derived from crystallographic studies); and (c) using siRNA-mediated Rac1 knockdown accompanied by rescue-experiment by wild-type Rac1. Involvement of Rac1 in the Nox1 system was supported by similar findings in two subsequent reports (17, 69) and two previous studies showing effects of RacG12V and RacGEF on Nox1 (55, 81). Recognition of involvement of Rac1 in the human Nox3 system had been controversial because of the predominant role of Noxo1 (98), however, two subsequent reports confirmed that Nox3 is Rac-dependent to the same extent that it involves Noxa1 (53, 93). As in the Nox2 system where the binding of Rac to $p67^{phox}$ is a critical regulatory point in Nox2 activation (112), the Rac-Noxa1 interaction likely occurs at the membrane in a GTP-dependent manner.

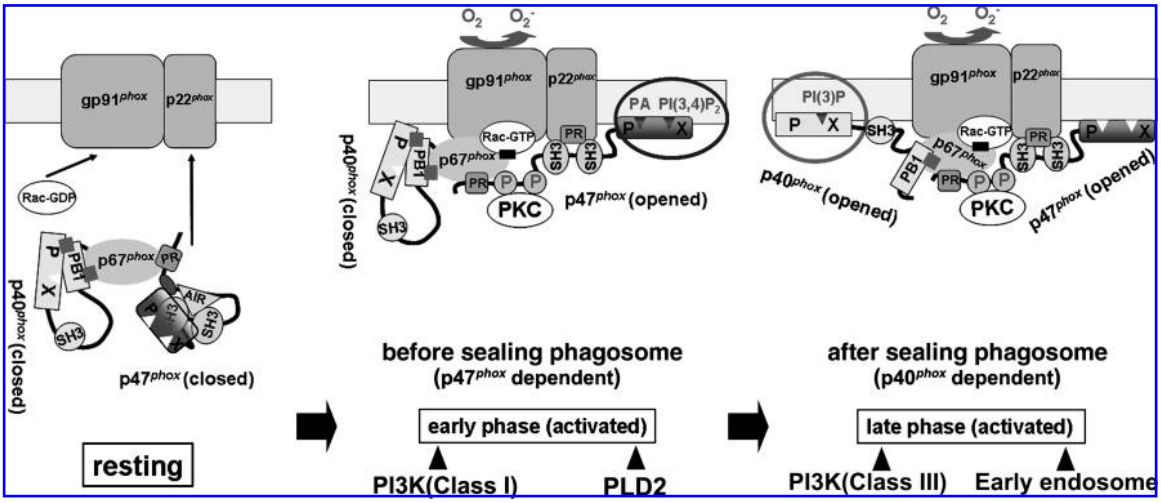

FIG. 5. Schematic representation of conformations changes in p47^{phox} and p40^{phox} allowing assembly of the ternary cytosolic complex into the active oxidase during the course of FcγR-mediated phagocytosis. The p47^{phox} component acts early-phase adaptor protein following PKC-dependent phosphorylation-induced conformational change by binding to p22^{phox} and membrane PA and PI(3,4)P_2. In contrast, p40^{phox} serves in the retention of the cytosolic complex on sealed phagosomes in later phases when PA and PI(3,4)P_2 have disappeared, due to its PX domain-PI(3)P and PB1 domain-p67^{phox} interactions (modified with permission from Fig. 11 of ref. 104). In the early phase, class I PI3-kinase (106) and PLD2 (15) serve in production of PI (3,4)P_2 and PA, while in the later phase class III PI3-kinase (106) and early endosomes (43) serve in accumulation of PI (3)P.

Three groups described four structural variants of human Noxo1 (α, β, γ, and δ) that arise from alternative mRNA splicing on both boundaries of the third exon, resulting in protein sequence variations within their PX domains (18, 95, 102) (Fig. 6). Based on their distinct tissue expression patterns, subcellular localization, and Nox1- or Nox3-supporting abilities, it appears that Noxo1β and Noxo1γ are the most physiologically relevant isoforms among the four variants (102). The isolated PX domains or full-length Noxo1β and Noxo1γ proteins are targeted to the plasma membrane, whereas the Noxo1α and Noxo1δ isoforms showed a tendency to aggregate in the cytoplasm or localized on intracellular vesicles (Fig. 7). Interestingly, Noxo1γ also localized in nucleus in several transfected cell models. Plasma membrane targeting of these variants correlated closely with their relative efficiencies in supporting oxidase activity (102).

PX domains are widely recognized as phosphoinositide lipid-binding modules in a variety of intracellular signaling proteins, although they also bind to some protein targets. As mentioned above, intramolecular protein contacts occur in both p47^{phox} and p40^{phox} involving their PX domains. The p47^{phox} PX domain has another remarkable feature of having a second anionic phospholipid-binding pocket with specificity for PA (54). Interestingly, an alignment of the p47^{phox} and Noxo1 PX domain protein sequences shows that residues involved in p47^{phox} PA binding are also conserved in Noxo1 (Fig. 6A). Crystallographic studies showed that the p47^{phox} residues lining the PA-binding pocket lie in α-helix1 and sequence preceding the PR motif: H51, K55, R70, P73, and H74 (Fig. 6B) (54); the corresponding aligned residues in Noxo1β and Noxo1γ are highly conserved: K49, K53, R68, P71, and K72 (Fig. 6A). Consistent with this feature, we showed that Noxo1β and Noxo1γ bind to PA, in addition to P1(4)P, P1(5)P, or PI(3,5)P_2 binding previously demonstrated by others (18, 95).

Based on the close structural similarities between p47^{phox} and Noxo1, we modeled the structures of Noxo1α, β, and γ-PX domains based on the solved structure of p47^{phox} (PDB ID: 1KQ6) and the alignment in Figure 6A using DeepView and SwissModel (http://swissmodel.expasy.org) (46). Sulfate and phosphatidylinositol molecules were added to the models by structural alignment with the p47^{phox} and p40^{phox} PX domains (PDB ID: 1H6H; Fig. 6B). The deletion of K50 in the Noxo1α isoform shifts the register of all amino acids in the second half of helix-1, which would drastically alter the overall character of the PX domain surrounding this highly conserved helix. This changes the composition of the PA binding site by replacing the lysine side chain with that of glutamate. These changes provide a structural explanation of the diminished ability of this isoform to support Nox1 activity and its tendency to aggregate (99). The 5-residue insertion of the Noxo1γ isoform is near the PA binding site, but it does not change the placement of critical binding residues when compared to the β isoform. These features provide a structural explanation for the preserved lipid and plasma membrane-binding activity of the γ isoform, despite the insertion. Further studies are needed to understand the basis for nuclear targeting of Noxo1γ and its role in supporting oxidase activity at this site.

Targeting and Activation of Duox Isozymes

Duox enzymes are expressed at highest levels on epithelial surfaces of mucosal tissues, such as major airways, the digestive and urogenital tracts, and several endocrine and exocrine glands, such as the thyroid, salivary glands, the pancreas, and the prostate (23, 28, 29, 31, 37, 42, 86). In polarized epithelium, the Duox proteins accumulate along the apical plasma membrane, where they release H_2O_2 to support several dedicated extracellular hemoperoxidases (14, 22, 31, 37, 86). In the thyrocyte, Duox2-dependent H_2O_2 generation activates TPO,

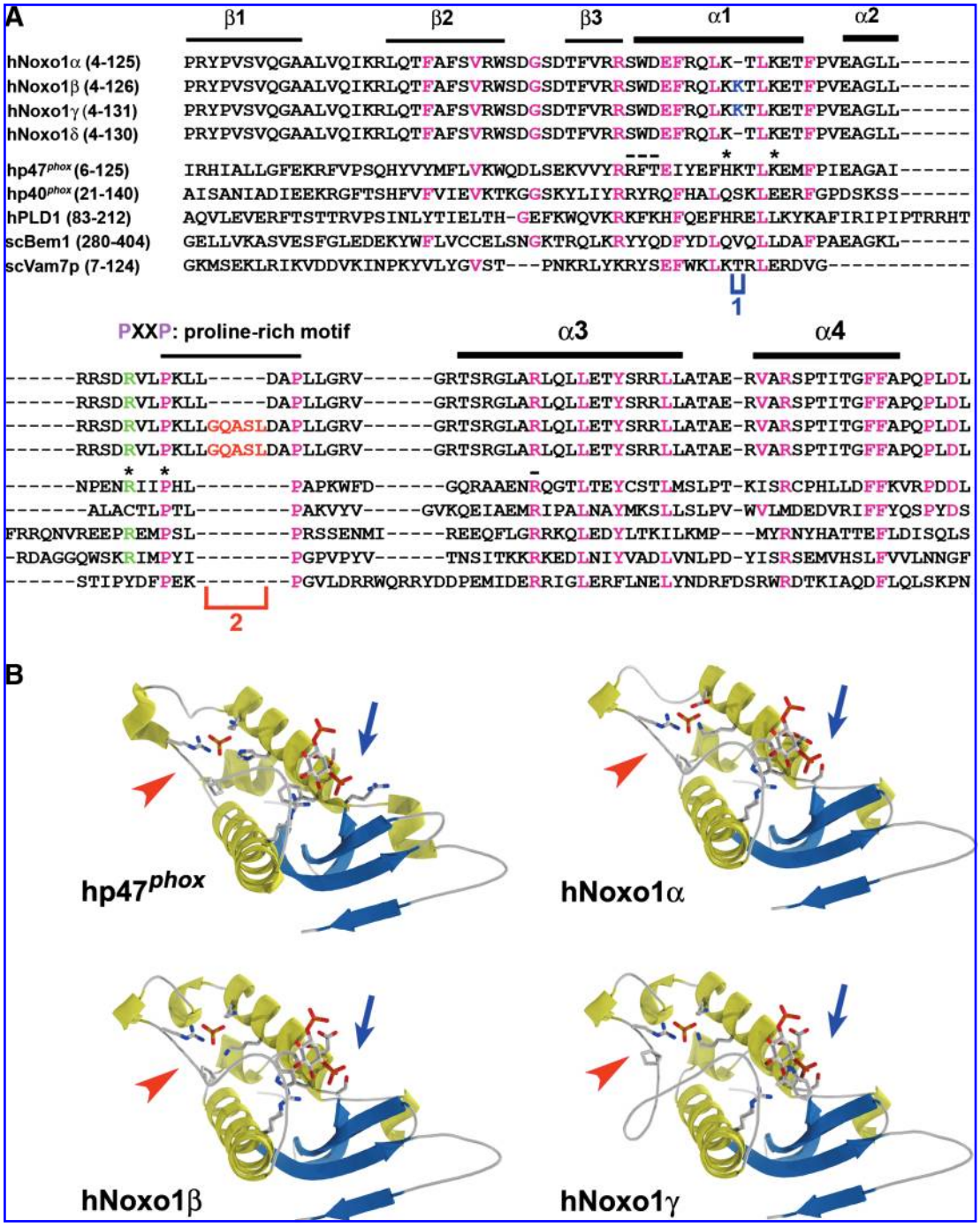

FIG. 6. Predicted three-dimensional structures of alternatively spliced PX domain variants of Noxo1, based on alignments with sequences of p47^{phox} and other PX domain-containing proteins. (**A**) Alignment of PX domain amino acid sequences, showing locations of splice site 1 (deleted from Noxo1α and δ) in the middle of conserved alpha-helix 1 and splice site 2 (present in Noxo1γ and δ) inserted within a variable loop region frequently containing the proline-rich motif in other PX domains. Conserved residues involved in p47^{phox} binding to PA are marked with an asterisk (*) while those that bind phosphoinositide lipids are *overlined* (modified with permission from Fig. 2 of ref. 102). (**B**) Structural models of the Noxo1 isoforms based on the structure of p47^{phox} (PDB ID: 1KQ6). The critical residues of the PA binding site are unchanged between the β and γ isoforms, despite the nearby insertion five amino acids in Noxo1γ. The electrostatic character of the PA binding site is significantly altered in the α isoform by the replacement of lysine by glutamic acid (*red arrow heads*). The PI binding site is basically unchanged between p47^{phox} and the Noxo1 isoforms (*blue arrows*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

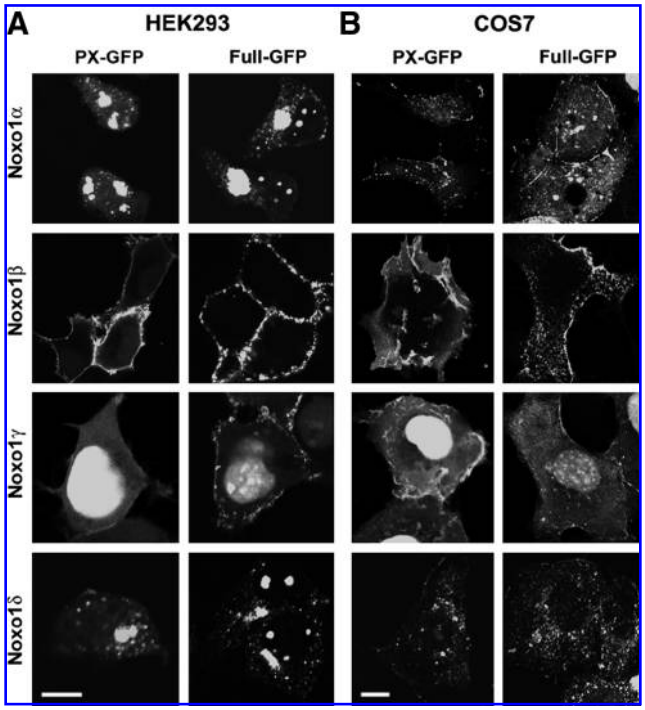

FIG. 7. Subcellular localization of PX domain and full-length Noxo1 alternative splice variants in HEK293 (A) and COS7 (B) cells. Noxo1α(PX)-GFP and Noxo1α-GFP show a tendency to aggregate or are localized on intracellular vesicles. Noxo1β(PX)-GFP and Noxo1β-GFP are localized predominately along the plasma membrane. Noxo1γ(PX)-GFP and Noxo1γ-GFP localize in the nucleus and along the plasma membrane. Noxo1δ(PX)-GFP and Noxo1δ-GFP show localization patterns similar to that of Noxo1α. Bar: 10 μm. (modified with permission from Fig. 2 of ref. 102).

which catalyzes T_3/T_4 hormonogenesis (74). In the lung and salivary glands, Duox provides H_2O_2 to LPO, which converts thiocyanate anions into the microbicidal oxidant hypothiocyanite (37, 42, 75, 84). Primary human bronchial epithelial cells maintained long-term on membranes in air–liquid interface cultures redifferentiate into a mature airway phenotype, resulting in significant upregulation of Duox1 expression (84). Immunostaining of these cultures demonstrates that Duox is localized on the apical aspect of the mature ciliated cells (Fig. 8). We and other groups showed these differentiated airway models produce sufficient Duox1-derived H_2O_2 from the apical surface to effectively support LPO and thiocyanate-mediated microbicidal activity against several airway pathogens (37, 75, 84).

Initial attempts at heterologous expression of the recombinant Duox enzymes failed to reconstitute these H_2O_2 generating systems. Immunostaining and glycosylation studies showed the newly synthesized Duox was blocked inside the ER, whereas thyrocyte Duox accumulates at the cell surface and bears Golgi-modified carbohydrates (22). Co-expression of $p67^{phox}$, $p47^{phox}$, $p22^{phox}$, or TPO did not rescue active Duox on the cell surface (22). Furthermore, inhibition of Rac by *C. difficile* toxin B had no effect on Duox activity (36). Using truncated Duox variants, a region encompassing the first transmembrane segment and the following 45 residues was shown to act as an ER retention signal (71). The missing Duox maturation factor was recently identified (44). Co-expression of the membrane protein Duox activator 2 (Duoxa2) enabled delivery of mature, glycosylated Duox2 to the cell surface, reconstituting oxidase activity (44). We cloned four alternatively spliced *DUOXA1* variants and compared their abilities to support Duox maturation (72). Two of the four Duoxa1 variants (Duoxa1β and δ) that lacked the third coding exon (shortening the first extracellular loops by 45 aa) were incompletely glycosylated and did not support Duox activity. Interestingly, we found both Duox enzymes could be rescued by Duoxa1α, Duoxa1γ, or Duoxa2 and detected both Duox/Duoxa proteins at the cell-surface (Fig. 9). Immunostaining of Duoxa1 in human airway sections also confirmed that Duoxa1 accumulates on the apical surface of the airway epithelium (72). These localization results contrast with the initial study, where Duoxa2 was proposed to be an ER resident even when co-expressed with Duox2 (44). Interestingly, depending on the Duoxa variant expressed, co-localization of Duox and the Duoxa proteins in our models showed distinct subcellular patterns: Duoxa1α and Duoxa2 were efficient in targeting Duox1 and Duox2, respectively, to the plasma membrane and reconstituting high levels of H_2O_2 generation. In contrast, Duox1 or Duox2 accumulate with Duoxa1γ within some intracellular compartment distinct from the ER, as well as the plasma membrane. With the exception of Duox2/Duoxa1α or Duoxa1γ combinations, the reconstituted Duox produced solely H_2O_2, not superoxide, and could be immunoprecipitated in a heterodimer complex with Duoxa on the plasma membrane (72, 111). These findings are consistent with previous observations on native Duox, where no O_2^- could be detected from the thyroid (4), lung (42), and sea urchin enzymes (47, 110). Thus, these maturation factors not only function in enabling Duox exit from the ER, they appear

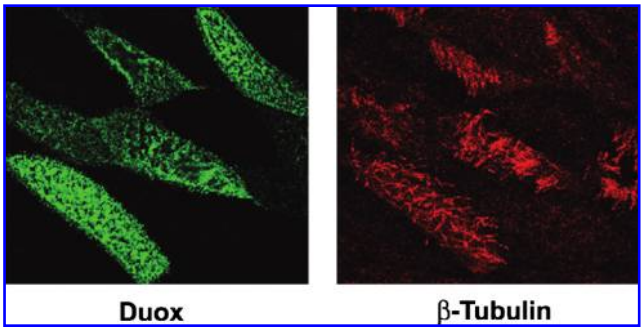

FIG. 8. Laser scanning confocal immunofluorescence detection of Duox on the apical plasma membrane surface of ciliated primary human bronchial epithelial cells. Images show the apical X-Y plane of confluent cells grown on permeable transwells and re-differentiated for 25 days in an air–liquid interface culture system, as described (84). Ciliated cells, stained with anti β-tubulin antibody (*red*), are the same as those stained positive for Duox (*green*). 3-D reconstructed images depicting the rotation of the X-Y plane are available in the supplemental online video, showing Duox and β-tubulin accumulation within the apical aspect of this polarized cell layer. (See online supplemental video at www.liebertonline.com). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

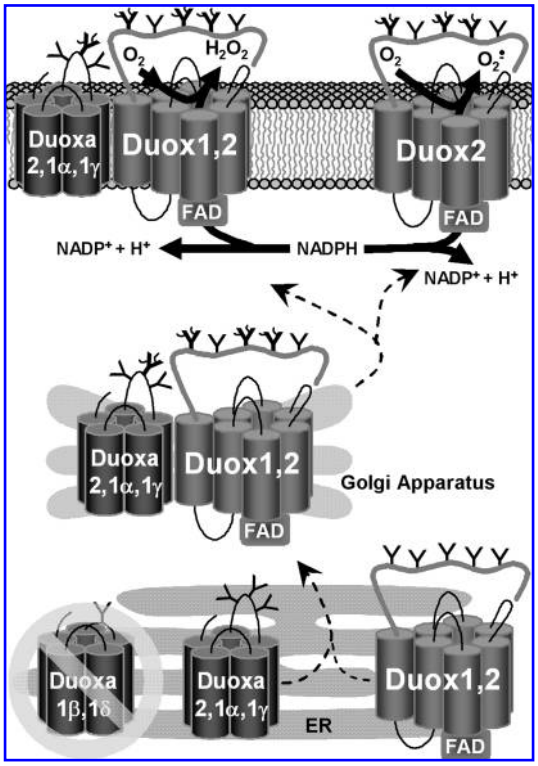

FIG. 9. Role of Duox maturation factors in Duox isozyme biosynthesis and ROS-generating mechanism. DuoxA2 and DuoxA1α have similar sizes and predicted N-glycosylation sites. Duoxa1γ has an extended C-terminal sequence similar to that of *Drosophila* NIP (numb interacting protein). Duoxa1β and δ forms (*bottom left*) that lack exon 3-encoded sequence and two predicted gylcosylation sites remain in the ER and do not support biosynthesis of active Duox. Active Duox maturation factors (Duoxa1α, γ, Duoxa2) translocate to the plasma membrane, undergoing Golgi-based carbohydrate modifications and forming stable H_2O_2-generating Duox/Duoxa complexes (*upper left*). Less competent combinations of Duox2 overexpressed with Duoxa1α or Duoxa1γ do not form plasma membrane Duoxa complexes, show less carbohydrate processing, and produce superoxide (*upper right*).

to co-translocate with Duox, undergo carbohydrate modifications in the Golgi apparatus, and affect the type of ROS produced by Duox (72).

The precise role of the Duox extracellular peroxidase homology domains has not yet been established. Contrary to its peroxidase-like designation, it does not appear to function as a peroxide-consuming core in Duox-reconstituted cells (72). Indeed, H_2O_2-dependent substrate oxidation by cell surface-exposed Duox required exogenous peroxidases (37, 44, 72, 84). In our hands, efficient Duox reconstitution correlated with cell surface exposure and always required an exogenous peroxidase to detect H_2O_2 production by oxidation of three different substrates (tyrosine, homovanillic acid, Amplex Red) (72). H_2O_2 formation most likely proceeds from formation of a superoxide intermediate by the Nox-like portion, with the peroxidase-like domains subsequently promoting intramolecular superoxide dismutation or other mechanisms leading to H_2O_2 release. Superoxide production could be measured from ER-blocked Duox2 in the absence of Duox maturation factors (4) or from surface-exposed Duox2/Duoxa1α or Duox2/Duoxa1γ combinations that do not form stable heterodimeric complexes (72, 111). In both cases, the Duox enzymes are incompletely processed in the absence of a stable Duox activator association. Thus, the Duox activators may function in processing of Duox isozymes into dedicated H_2O_2 generators or they could even serve as part of H_2O_2-forming Duox complexes (72).

Exactly where in the cell Duox becomes an active enzyme remains unclear, that is, within the Golgi apparatus or after reaching the plasma membrane. In reconstituted HEK293 cells, Duox reconstitution correlated closely with Golgi-based modification in both Duox and their maturation factors (72). As robust generators of toxic ROS, Duox enzymes may be designed for activation only on the cell surface, releasing high amounts of these products outside of the cell. Thyroid H_2O_2 is detected only on the apical pole, but not inside the cell (11, 30, 70). In other settings, lower levels of Duox activity may serve other functions within intracellular sites, such as cell signaling involved in migration or proliferation (i.e., would healing) (109), perhaps under the control of distinct targeting, maturation, or regulatory factors.

In conclusion, the diverse Nox family of NADPH oxidases serves a variety of essential roles requiring deliberate ROS generation in many tissues. Each of these enzymes is subject to unique mechanisms that control the site, duration, amount, and type of ROS generated to avoid collateral oxidative damage to host tissues. It has been known for years that the multicomponent Nox2-based oxidase is subject to tight regulation in order to direct potent microbicidal oxidants to the phagosomal compartment. Recent studies provide new insights on $p40^{phox}$-based regulation of this system. Other closely related multicomponent oxidases, Nox1 and Nox3, are subject to similar mechanisms controlling the biosynthesis and sites of assembly and activation, involving participation of $p22^{phox}$, Rac, and similar organizer and activator proteins. In contrast, the dual oxidases are specialized H_2O_2 generators that have developed distinct maturation, subcellular targeting, and activation mechanisms, enabling calcium-triggered ROS release from the cell surface to support several tissue-specific extracellular peroxidases.

Acknowledgments

This work was supported by the National Institutes of Health Intramural Research Program, National Institute of Allergy and Infectious Diseases, and Grant-in-Aid for Scientific Research on (C), on Priority Areas, and on the Global-COE Program in Japan. We thank to Dr. Corinne Dupuy, Institut Gustave Roussy, Villejuif, France for kindly providing anti-Duox antibody.

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Date of first submission to ARS Central, April 21, 2009; date of acceptance, May 13, 2009.

Abbreviations Used

AIR = auto-inhibitory region
CGD = chronic granulomatous disease
Duox = Dual oxidase
Duoxa = Duox activator
ER = endoplasmic reticulum
Nox = NADPH oxidase
PA = phosphatidic acid
PB1 = Phox and Bem1
phox = phagocytic oxidase
PX = phox
ROS = reactive oxygen species
SH3 = Src homology 3

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