NOXO1: Regulation of Lipid Binding, Localization and Activation of Nox1 by the PX Domain

Guangjie Cheng and J. David Lambeth+

Department of Pathology and Laboratory Medicine
Emory University School of Medicine
Atlanta, Georgia, 30322 USA

Running title: PX Domain of NOXO1

+To whom correspondence should be addressed
NOXO1 (Nox organizing protein 1) and NOXA1 (Nox Activating protein 1) are homologs of p47phox and p67phox. p47phox functions in phagocytes as an essential organizing protein mediating the binding of other regulatory proteins during activation of the phagocyte oxidase, and its translocation to the membrane is triggered upon cell activation by hyperphosphorylation, which relieves autoinhibition of SH3 and PX domains. NOXO1 lacks an autoinhibitory region and phosphorylation sites that are present in p47phox. Co-transfection of Nox1, NOXO1 and NOXA1 reconstitutes ROS (reactive oxygen species) generation in HEK 293 cells in the absence of cell stimulation. NOXO1 binds to the phosphatidylinositol (PtdIns) lipids PtdIns 3,5-P2, PtdIns 5-P and PtdIns 4-P. Unlike p47phox, which is located in the cytosol of resting cells and translocates to the plasma membrane where gp91phox is located, NOXO1 co-localizes with Nox1 in the membranes of resting cells. This localization of NOXO1 is dictated by its PX domain, since this domain but not the remainder of the molecule localizes to membranes. A point mutation in the PX domain of holo-NOXO1 decreases lipid binding resulting in cytosolic localization, and also inhibits NOXO1-activation of Nox1. Thus, in transfected HEK 293 cells, NOXO1 and NOXA1 activate Nox1 without the need for agonist activation, and this is mediated in part by binding of the NOXO1 PX domain to membrane lipids.
Introduction

A family of reactive oxygen-generating enzymes with homology to gp91phox, the catalytic subunit of the phagocyte NADPH-oxidase, was recently described (1)(2-4). Gp91phox contains FAD, two hemes and an NADPH binding site, and together with p22phox constitutes flavocytochrome b₅₅₈, an intrinsic membrane complex that constitutes the core of the phagocyte oxidase. The oxidase is activated by immune mediators and by bacterial products and participates in the first line of host defense, generating large amounts of reactive oxygen species (ROS) that function directly (5) and indirectly (6) to kill the invading microbe. In contrast, the Nox enzymes are expressed in tissues that are not typically thought of as participating in host defense, and other functions have been proposed including regulation of cell growth, apoptosis and angiogenesis (1,7-9). In addition, inappropriate activation or expression of Nox enzymes, particularly Nox1, has been associated with neoplastic growth including cancer (9)(Arnold, R.S., unpublished) and atherosclerosis (10,11). Similarly, ROS generation through Nox enzymes may be relevant to insulin mechanisms and hence diabetes (B. Goldstein, personal communication). Therefore, characterization of the mechanisms by which Nox enzymes regulate ROS production may be relevant to the pathophysiology of these diseases.

In naïve neutrophils, the phagocyte flavocytochrome b₅₅₈ is dormant, and is activated upon cell stimulation by the assembly with regulatory subunits present in the cytosol, reviewed in (12,13). These include p47phox, a 47 kDa protein, p67phox, a 67 kDa protein, and Rac (Rac2 and possibly Rac1, closely related isoforms). The proteins
p47phox and p67phox exist in resting cells in a cytosolic complex with a third regulatory protein, p40phox, which may function in either inhibiting or stimulating oxidase activity. Assembly of p47phox and p67phox with flavocytochrome b₅₅₈ is triggered by hyperphosphorylation of p47phox (14,15) and phosphorylation of p67phox (16,17) via several kinases including protein kinase C (18) and Akt (19).

Phosphorylation of p47phox occurs in an autoinhibitory domain (AID) which in the absence of phosphorylation, binds its own tandem SH3 domains (20-22), preventing interaction with p22phox. The latter, in addition to forming a heterodimer with gp91phox (23,24), provides a proline-rich binding site for the p47phox bis-SH3 domain. p47phox also contains a lipid binding domain (the PX domain) with specificity for 3-phosphorylated lipids, the products of phosphatidylinositol 3-kinase (25-29). The latter is activated in cells exposed to bacterial products or immune mediators, as are the kinases that phosphorylate p47phox itself. The binding of the PX domain is masked by the C-terminal SH3 domain of p47phox, preventing its interaction with lipids until the autoinhibitory domain becomes phosphorylated and the SH3 domains bind to p22phox. Thus, upon cell activation, several pathways converge on p47phox, relieving autoinhibitory constraints and allowing it to interact with both membrane lipids and with p22phox. This provides one of the important triggers for assembly of the phagocyte oxidase, and autoinhibitory constraints within p47phox prevent ROS generation in the absence of immune mediators or agonists.
Homologs of p47phox and p67phox have recently been identified independently by several groups (31-33) including ours (GenBank # AF 532984). The p47phox homologue is referred to as NOXO1 (NOX Organizing Protein 1), based upon the prediction that it will have a function analogous to p47phox. The p67phox homolog is called NOXA1 (Nox Activating Protein 1), based on the concept that the p67phox is the immediate regulator of electron transfer within gp91phox. These investigators also demonstrated using transfection that high level ROS generation by Nox1 requires both NOXO1 and NOXA1. In addition, Takeya et al. (32) showed that the system requires p22phox for optimal activity, and that NOXO1, via its tandem SH3 domains, can form a complex with p22phox, suggesting that it performs the same role for Nox1 as it does for gp91phox, providing a docking site for the organizing protein.

Like p47phox, NOXO1 contains an N-terminal PX domain (PX refers to Phox Homology). This domain, present in a variety of proteins, participates in protein-lipid (28) and protein-protein interactions (30,34). In contrast to p47phox, NOXO1 participates in activation of Nox1 in the absence of cell activation vide infra and (31-33). NOXO1 lacks the autoinhibitory domain and phosphorylation sites that are present in p47phox. The present study was undertaken to elucidate the function of the PX domain of NOXO1, including its lipid binding specificity, its role in activation of Nox1 and its role localization of NOXO1. Our results implicate the NOXO1 PX domain as an important determinant of both membrane localization and activity. The interaction of this domain with lipids characteristic of resting cells provides a rationale for the ability of NOXO1 to activate Nox1 in the absence of agonists.
EXPERIMENTAL PROCEDURES

Cells and Reagents.

HEK293H cells (Invitrogen, Carlsbad, CA) were maintained in DMEM with 10% fetal bovine serum (FBS), 100 U penicillin, and 100 µg streptomycin. Luminol, glutathione, isopropyl-β-D-thiogalactopyranoside (IPTG), and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (St. Louis, MO), Hanks’ solution containing 1.26 mM calcium and 0.91 mM magnesium from Invitrogen (Carlsbad, CA), pEGFP-endo and DNA polymerase from Clotech (Clontech, Palo Alto, CA), restriction endonucleases and ligase from New England Biolabs (Beverly, MA), glutathione-Sepharose 4B from Amersham Biosciences (Piscataway, NJ), and Topro-3 from Molecular Probes (Eugene, OR). E39 monoclonal antibody (the kind gift of Dr. Glenn Pilkington and colleagues at Diadexus) was produced by immunization of BALB/c mice with Nox1(235-248), representing the predicted third extracellular loop of the transmembrane domain, conjugated to KLH. Hybridomas were initially screened by ELISA using the same peptide conjugated to BSA, and using the gp91phox ectodomain peptide as a negative control. Peptides were synthesized by SynPep (Dublin, CA). E39 monoclonal antibody showed bright indirect immunofluorescence in NIH 3T3 cells expressing Nox1, but no fluorescence in control NIH 3T3 cells. Nox1-expressing, but not control NIH 3T3 cells showed a 62 kDa band by Western blotting. Anti-NOXO1 antibody was an IgY fraction generated in chicken and purified from egg yolk by Lampire Biological Laboratory (Pipersville, PA), using KLH-conjugated peptide NOXO1(353-370). This peptide was synthesized and purified by reverse phase HPLC by Emory’s Microchemical Facility.
Molecular cloning of the cDNA of NOXO1 and NOXA1.

We previously reported the cDNA sequence for human NOXO1\textsuperscript{a} (AF532984, deposited July 29, 2002). NOXO1\textsuperscript{a} is the major splice form present in colon (G. Cheng, unpublished), and is used in all experiments in the present studies. The details of the molecular cloning of NOXO1 and NOXA1 will be detailed in a separate communication (Cheng and Lambeth, manuscript in preparation). All cDNA clones were sequenced in both strands using an ABI Model 377 sequencer. The cDNA cloning of Nox1, Nox3 and Nox4 was described previously (1,3). The cDNA for gp91\textsuperscript{phox} was subcloned into pcDNA3.1 from EST clone AA996283 and the cDNA was verified as above. All other cDNA constructs were made in pcDNA3 except for NOXA1, which was in pCMV-Sport6.

Subcloning.

Human NOXO1, NOXO1(1-147), and NOXO1(148-371) were expressed in-frame in pDsRed2 C1 (Clontech, Palo Alto, CA) to generate NOXO1 domains fused at their N-termini with the red fluorescent protein DsRed2. NOXO1(R40Q) was generated using a primer (5'-GCCTGAATTCGTCCCAACTCTGGCGCACGAAGGTGTCGC-3') containing a point mutation and the naturally occurring 3'EcoR I site. This was used with a 5' corresponding to the vector sequence and containing a Kpn1 site. The PCR product was subcloned into the pDsRed2 C1 plasmid using EcoR I and Kpn 1 restriction enzymes. For localization studies, h-Nox1 and p22\textsuperscript{phox} were subcloned in pEGFP-N1 (Clontech, Palo Alto, CA) to generate Nox1 or p22\textsuperscript{phox} fused at their C-termini with EGFP. Nox1(P409H) in pcDNA3.1 was generated by amplifying Nox1 in pcDNA3.1
using primer 1 (5’-TTGGGGTCAACCCACTTTGCTTCTATCTTTGAAATCC-3’) and primer 2 (5’-GGGAAGCTTTCAAAAATTTTCTTTTTGTTGAAGTA-3’). Primer 1 encodes the P409H mutation (italics) and a BstE II site (underlined, originally present in Nox1) and primer 2 introduces a Hind III site (underlined). Using these restriction sites, the amplified fragment replaced the same excised region of Nox1 in pcDNA3.1. Using a similar strategy, NOXO1(1-147) and NOXO1(1-147)R40Q were subcloned into pGEX-4T-3 (Amersham Biosciences, Piscataway, NJ) to generate GST fusion protein with the GST.

**Overexpression and purification of the PX domain of NOXO1.**

Plasmids encoding GST-NOXO1(1-147) or GST-NOXO1(1-147)R40Q were transformed into *E. coli* strain BL21 (DE3) Gold (Stratagene, La Jolla, CA) and cultures were grown at 22°C to an OD$_{600}$ of 0.6. Protein expression was induced for 3 hrs at 22°C with 0.2 mM IPTG. Fusion proteins were purified on glutathione-Sepharose 4B resin according to the manufacturer’s instructions. The purified fusion proteins were dialyzed against PBS at 4°C with two buffer changes. Protein concentration was measured using Protein Assay (Bio-Rad, Hercules, CA).

**Phospholipid binding.**

Lipid binding studies were carried out using the PIP array™ from Molecular Probes (Eugene, OR) following the manufacturer’s protocol. Membranes were first incubated with 3% fatty acid-free BSA /TBS-T buffer at room temperature x 1h and then overnight at 4°C with 100 ng/ml of GST-fusion protein. After washing three times with TBS-T
buffer, the membranes were incubated with 1µg/ml anti-GST monoclonal antibody (Cell Signaling Technology, Beverly, MA). Membranes were further incubated with 1: 5000 rabbit anti-mouse antibody conjugated to horse radish peroxidase, and chemiluminescence was developed using SurperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and recorded using X-ray films.

**Transient transfections.**

pcDNA3 (empty vector or vector harboring Nox1, NOXO1 and/or NOXA1 alone or in combination) were transfected into HEK 293H cells using Fugene 6 (Roche, Indianapolis, IN) according to manufacturer’s instructions. After 48 hrs, cells were removed from the well and washed twice with cold Hanks’ solution containing calcium and magnesium.

**Intracellular Localization of NOXO1.**

HEK 293H cells grown to ~40% confluence on cover slips were transfected with control or recombinant pDsRed2 plasmid (0.5 µg each) expressing fluorescent fusion proteins of Nox1 or NOXO1. After 48 hr, the cells were fixed in 4% paraformaldehyde x 15 min. and were stained with 1 µg/ml Topro-3 for 10 min to visualize nuclei. Fluorescence images were recorded using a Zeiss Axioplan 2 LSM 510 Meta Laser Scanning Confocal Microscope System. Immunofluorescence was carried out after cotransfection with vectors encoding Nox1 and NOXO1 holoproteins. After 48 hr, cells were fixed as above and incubated with 3% BSA/PBS at room temperature x 1 hour. After extensive washing, cells were incubated in 3% BSA/PBS buffer containing 2.83 µg/ml of E39 anti-Nox1
monoclonal antibody plus 3.0 µg of anti-NOXO1 chicken polyclonal antibody for 3 hours. After washing, cells were incubated in 3% BSA/PBS buffer containing 2 µg/ml of Alexa FluorR488 goat anti-mouse IgG and 2 µg/ml of Alexa FluorR594 goat anti-chicken IgG for 1 hr. Fluorescence images were recorded by confocal microscopy as above.

Measurement of Reactive Oxygen.

Reactive oxygen was measured with luminol luminescence, essentially according to (35). Briefly, for each well in a 96-well plate, 5 x 10^5 cells in Hanks’ buffer with calcium/magnesium were mixed with 200 µM Luminol plus 0.32 units of HRP in a 200 µl total volume. Luminescence was quantified using a LumiCount™ luminometer (Packard, Meiden, CT) and luminescence was recorded every minute for 1 hr.

RESULTS

Reconstitution of Nox1-Dependent Reactive Oxygen Generation by NOXO1 and NOXA1. In Fig. 1, Nox1-dependent activity was reconstituted by co-transfecting HEK293 cells with Nox1, NOXO1 and NOXA1. In Panel A, Nox1 was always present, and cells were co-transfected with empty vector, or plasmid encoding NOXO1 or NOXA1 individually or in combination. As is shown, activity required the presence of both NOXO1 and NOXA1, since when either was omitted, only extremely low basal activity was seen. In Panel B, NOXO1 and NOXA1 were always present (introduced by co-transfection), and the concentration of plasmid encoding Nox1, Nox1(P409H) or empty vector was varied. Data demonstrate that high levels of ROS generation are
achieved using Nox1 but not Nox1(P409H) which is mutated in the predicted NADPH binding site. This mutation corresponds to an inactivating mutation in gp91phox that is present in one variant of Chronic Granulomatous Disease (36,37). In an earlier publication (1), we observed low level ROS generation in fibroblasts transfected with Nox1 alone. We have detected low levels of NOXO1 and NOXA1 mRNA in these cells (R.S. Arnold, unpublished), and this may account for the low ROS generation seen in these cells when Nox1 was introduced. Thus, these data confirm studies that appeared during the preparation of this manuscript (31-33) that concluded that activation of Nox1 requires the regulatory proteins NOXO1 and NOXA1.

**Co-localization of Nox1 and NOXO1 in Transfected Cells.** Nox1 and NOXO1 were expressed in HEK293 cells as fusion proteins with Green Fluorescent Protein (GFP) and a red fluorescent protein (DsRed2). As shown in Fig. 2, left upper panel, Nox1 is expressed in plasma membrane and in additional large intracellular structures that co-localize (data not shown) with an endosomal marker (EGFP-endo) within the cytosolic compartment, and may therefore represent fused endosomes. The red fluorescent version of NOXO1 (Fig. 2, middle upper panel) appears to be located in both plasma membrane and in these structures, and the yellow merged image (Fig. 2, right upper panel) confirms that the two proteins co-localize. The location of these components did not change following treatment of cells with the protein kinase C activator phorbol 12-myristate, 13-acetate (10 nM, data not shown). In Fig. 2, lower panels, a similar experiment was carried out, co-transfecting NOXO1-DsRed and p22phox-GFP in HEK293 cells. As shown, the NOXO1 and the p22phox co-localized to the same structures, which include
plasma membrane and internal membranes similar to those seen in the upper panels. In co-transfection experiments such as those shown in Fig. 1, the NOXO1 fusion protein was active in supporting ROS generation, whereas the Nox1-GFP fusion protein was inactive (data not shown).

Because the partial endosomal location may have represented an artifact of overexpression of the unnatural fusion proteins and because the Nox1-GFP fusion protein was inactive, we also carried out immunofluorescence studies using mouse and chicken antibodies specific for Nox1 and NOXO1. The latter were transfected into HEK293 cells as non-fusion proteins. As shown in Fig. 3, Nox1 and NOXO1 were both expressed in plasma membrane. In addition, a small percentage of NOXO1 was expressed in punctate structures throughout the cell, but neither protein was expressed in the fused endosome-like structures seen when fluorescent fusion proteins were expressed. When the identical immunostaining procedure was carried out using non-transfected cells, no immunostaining was seen (lower panels in Fig. 3). Thus, both immunostaining and fluorescent fusion proteins indicate a plasma membrane localization in transfected HEK293 cells. The plasma membrane (or phagosomal membrane) corresponds to the location of gp91phox and p47phox in activated phagocytes. Membrane co-localization of these proteins in unstimulated cells is consistent with the finding that co-transfection results in activity in the absence of an added agonist such as phorbol ester.

**Lipid Binding of the NOXO1 PX Domain.** The PX domains of NOXO1 [NOXO1(1-147)], the same construct in which arginine 40 was mutated to a glutamine, or the PX
domain of p47phox [p47phox(1-144)] were expressed in *E. coli* as fusion proteins with glutathione S-transferase. Fusion proteins were purified to obtain homogeneous protein (Fig. 4, Panel A). Lipid binding was then measured using overlay blotting as in Experimental Procedures (Fig. 4, panel B). The data show significant binding to phosphatidylinositol 3,5-bisphosphate, phosphatidylinositol 5-phosphate, and phosphatidylinositol 4-phosphate, with minor binding to phosphatidylinositol 3,4-bisphosphate. Notably, there is strong binding to mono phosphorylated phosphatidylinositols that are present in resting cells (38). Mutation of arginine 40 is predicted based on analogous mutations in p47phox and p40phox (27) to disrupt lipid binding. This is confirmed in Panel C, which shows that this mutation interferes with the ability to bind to phosphatidyl inositol phosphate lipids. In contrast to the specificity of the NOXO1 PX domain, the PX domain of p47phox bound most tightly to phosphatidylinositol 3,4-bisphosphate and to a lesser extent, phosphatidylinositol 3-phosphate (Panel D, Fig. 4), as reported previously (27).

**A Mutation Affecting Lipid Binding Decreases the Ability of NOXO1 to Activate ROS Generation.** Cells were co-transfected with empty vector or plasmid encoding Nox1, NOXA1, plus either NOXO1 or NOXO1(R40Q). Figure 5 shows that NOXO1(R40Q) is considerably less potent in activating Nox1-dependent ROS generation than is wild type NOXO1.

**Lipid Binding by the PX Domain of NOXO1 Localizes the Protein to Plasma Membrane.** Plasmids encoding NOXO1 fusion proteins with the red fluorescent protein
DsRed2 were constructed using either the holoprotein NOXO1(1-371), the PX Domain NOXO1(1-147) or the remainder of the molecule NOXO1(148-371). The latter region contains a tandem SH3 region that is predicted to participate in protein interactions. Fig. 6 demonstrates that while the DsRed2 alone localizes throughout the cell, when it is fused to holo-NOXO1, it localizes as in Fig. 2 and Fig. 3 to plasma membrane and to some internal membrane structures within the cytosolic compartment. Fig. 6 also shows that when DsRed2 is fused to NOXO1(1-147), the PX domain, it localizes to the same membrane structures/compartments as does the holoprotein. However, NOXO1(148-371), which lacks the PX domain but which retains the tandem SH3 domains that in p47phox bind to p22phox, localizes throughout the cell in a pattern similar or identical to that of the non-fused DsRed2 protein. Using both RT-PCR and Western blotting, p22phox message and protein, respectively, were seen in HEK 293 cells (data not shown). Thus, the PX domain in NOXO1 contributes significantly to the membrane localization of NOXO1.

Consistent with this interpretation, the DsRed2 fusion protein of holo NOXO1(R40Q) fails to localize in the plasma membrane, and shows a distribution throughout the cell, similar to that of DsRed alone (Fig. 7). Thus, the same mutation in NOXO1 that disrupts lipid binding also interferes with plasma membrane binding in intact cells.
DISCUSSION

PX domains were described as a conserved domain found in more than 100 eukarotic proteins including p47phox, p40phox, phospholipase D, the Cpk class of phosphatidylinositol 3-kinases, sorting nexins, and Bem1 and Scd2 from yeast (39). The PX domain of p47phox contains a proline-rich sequence that appears to participate in intradomain binding to the C-SH3 domain of p47phox (22,26). Moreover, PX domains function as binding sites for phosphatidylinositol phosphates (25,27,29,40). The internal interactions within p47phox mask the binding to lipids, preventing membrane association. When the autoinhibitory domain becomes hyper-phosphorylated by kinases such as protein kinase C and Akt releases inhibition, permitting binding of the bis-SH3 domain to p22phox and allowing the PX domain to bind to lipids (22,30).

The PX domain of p47phox and p40phox bind to the lipid products of phosphatidylinositol 3-kinase, showing the greatest affinity for PtdIns 3,4-bisphosphate. Phosphatidylinositol 3-kinase is a regulated lipid kinase that in phagocytes is activated by the same inflammatory stimuli that activate reactive oxygen generation (41). The p47phox PX domain contains a second lipid binding site that interacts with phosphatidic acid, providing a synergistic binding to inositol lipids and phosphatidic acid (30,42). Phosphatidic acid is the product of phospholipase D, which is also activated in phagocytes in response to stimuli that activate the phagocyte NADPH-oxidase (43,44). Thus, the p47phox possesses binding sites that respond to lipids that are produced during cell activation. In contrast, NOXO1 binds with high affinity to the mono-phosphorylated
phosphatidylinositols PtdIns 4-P and PtdIns 5-P, which are present as constituents of plasma membranes in cells that have not been exposed to activating stimuli (38).

Thus, unlike p47phox, NOXO1 is predicted based on its phospholipid binding specificity to interact with plasma membranes in cells that have not been stimulated with an agonist. This prediction is borne out in the present studies, which show that NOXO1 binds to membranes of resting cells, including the plasma membranes. This membrane binding localizes NOXO1 in the same membranes where Nox1 is located. A mutation in the predicted lipid binding site of NOXO1 interferes with lipid binding, and disrupts co-localization with Nox1 in both the PX domain itself and the holo-NOXO1. Holo-NOXO1 contains a tandem SH3 region that in p47phox interacts with p22phox. Similarly, p22phox has recently been shown to be required for Nox1-supported ROS generation (32). However, herein we show that even though p22phox, Nox1 and NOXO1 co-localize to the same membrane compartments, mutation of the lipid binding domain of NOXO1 interferes with co-localization, releasing NOXO1 into the cytosol. Thus, the SH3 domains alone are not sufficient to assemble NOXO1 with NOX1/p22phox. The same lipid binding site mutation also decreases the ability of NOXO1 to support Nox1-dependent ROS generation, consistent with an effect on localization. An analogous mutation in the lipid binding site in p47phox failed to interfere with binding to the plasma membrane (29), indicating that for p47phox, association with other biomolecules (such as p22phox) may provide significant binding energy which cooperates with lipid binding in localizing p47phox to the appropriate compartment. The tandem SH3 domain in p47phox binds tightly to p22phox, and following phosphorylation-induced release from inhibition
by the autoinhibitory domain, may provide significant binding energy for assembly of the phagocyte oxidase. While p22phox is important for the function of Nox1 (32), the present studies show that in HEK 293 cells, the interaction with the tandem SH3 domains of NOXO1 is not sufficient by itself to localize NOXO1 to the same compartment as Nox1. These data point to the importance of the PX domain of NOXO1 in localizing this subunit to the same membranes wherein Nox1 is located, accounting in part for the constitutive activity of Nox1 in the absence of agonist stimulation of cells. These data do not eliminate the possibility that Nox1 may be further activated in agonist-simulated cells. Indeed, recent studies point to the involvement of Nox1 in PDGF and Angiotensin II-stimulated ROS generation in vascular smooth muscle cells (1,45). In addition, other groups have noted partial activation of Nox1 in other cell lines (32,33), suggesting that its activity may be partially consitutive and partially activatable. In our experiments (Cheng, unpublished), in HEK293 cells, addition of phorbol ester results in a 20-50% increase in ROS generation, while in COS7 cells, there is a ~2-fold increase in activity. Thus while there is significant consitutive activity in cell lines tested thus far, the magnitude of further activation by phorbol ester is cell-type specific. Such activation may involve the participation of an additional activatable component such as Rac or some as-yet uncharacterized component. Alternatively, in some cases, such activation might involve the participation of p47phox, which has recently been implicated in vascular ROS generation. However, transfection experiments have not thus far supported a major role for p47phox in Nox1 regulation (32,33). Thus, Nox1 might be regulated both by constitutive mechanisms involving NOXO1 and by acutely agonist-regulated mechanisms involving other components. Such mechanisms would provide the cells a
means by which both constitutive ROS levels could be maintained (using NOX01 expression) and agonist-specific ROS elevation could still be achieved.

References


Fig. 1. Co-transfection of Nox1, NOXO1, and NOXA1 results in high level ROS Generation. Panel A. Human NOXO1, h-NOXA1 or empty vector were co-transfected (0.5 µg plasmid each) along with h-Nox1 (0.2 µg plasmid) into HEK 293H cells. After 48 hrs, ROS production was measured using luminol luminescence as described in Experimental Procedures. Panel B. NOXO1 and NOXA1 plasmid DNA concentration was held constant (0.5µg each), and the plasmid encoding Nox1 or Nox1(P409H) was varied as indicated, keeping the total vector concentration constant using empty vector.
Fig. 2. Co-localization of Nox1 and NOXO1. In the top panels, Nox1 and NOXO1 (the holoproteins) were co-expressed as fusion proteins with green (GFP) and red fluorescent proteins (RFP, DsRed2), respectively, and were visualized by confocal microscopy. In the bottom panels, p22phox and NOXO1 holoproteins were co-expressed as green and red fluorescent fusion proteins, as above. The merged yellow to orange images at right indicates co-localization.
Fig. 3. **Colocalization of Nox1 and NOXO1 by immunofluorescence.** Nox1 and NOXO1 holoproteins were co-expressed in HEK 293 cells, and cells were fixed and stained with E39 monoclonal antibody against Nox1 or with a chicken polyclonal antibody against NOXO1 as indicated. In the lower panels, non-transfected HEK293 cells were stained with the same antibodies. After incubation with fluorescent-labeled second antibodies, immunofluorescence was visualized using confocal microscopy as described in Experimental Procedures.
Fig. 4. **Binding of NOXO1 PX domain to phosphatidylinositol lipids.** The GST fusion proteins of NOXO1(1-147) and NOXO1(1-147)R40Q were expressed in *E. coli* and purified on glutathione-Agarose (Panel A). Arrows in panel A correspond to molecular weight markers of 50, 40 and 25 kDa (top to bottom). The purified proteins were used in overlay lipid binding assays [NOXO1(1-147), Panel B; and NOXO1(1-147)R40Q, Panel C] performed as described in **Experimental Procedures.** Panel D shows lipid binding by the p47*phox* PX domain (residues 1-144).
Fig. 5. A mutation in the NOXO1 PX Lipid Binding Domain Reduces Its Ability to Activate Nox1. Cells were co-transfected with h-Nox1 (0.2 µg plasmid), h-NOXA1 (0.5 µg plasmid) and ether empty vector, NOXO1, or NOXO1(R40Q) (0.5 µg plasmid DNA each), and ROS production was monitored using luminol luminescence. Error bars show the range of two determinations in a single experiment, and the experiment shown was repeated twice.
Fig. 6. The PX Domain of NOXO1 determines membrane localization. 0.5 μg DNA of DsRed2 plasmid alone, NOXO1(1-371)-DsRed2 (holoprotein), NOXO1(1-147)-DsRed2 (PX Domain), or NOXO1(148-371)-DsRed2 (remainder of the molecule including tandem SH3 domains) were expressed in HEK 293 cells and visualized by confocal microscopy.
Fig. 7. **Effect of mutation of the lipid binding site on localization of holo-NOXO1.** The holoprotein forms of NOXO1 or NOXO1(R40Q) were expressed as DsRed2 fusion proteins in HEK293 cells, and cells were visualized using confocal microscopy. Nuclei are stained with Tropo-3.
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Guangjie Cheng and J. David Lambeth

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