Point Mutations in the Proline-Rich Region of p22phox are Dominant Inhibitors of Nox1- and Nox2-Dependent Reactive Oxygen Generation

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Running title: p22phox mutants as Nox inhibitor

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The integral membrane protein p22phox is an indispensable component of the superoxide-generating phagocyte NADPH oxidase whose catalytic core is the membrane-associated gp91phox (a.k.a. Nox2). p22phox associates with gp91phox and, through its proline-rich C-terminus, provides a binding site for the tandem SH3 domains of the activating subunit p47phox. While p22phox is expressed ubiquitously, its participation in regulating the activity of other Nox enzymes is less clear. This study investigates the requirement of p22phox for Nox enzyme activity and explores the role of its proline-rich region (PRR) for regulating activity. Co-expression of specific Nox catalytic subunits (Nox1, Nox2, Nox3, Nox4, or Nox5) along with their corresponding regulatory subunits (NOXO1/NOXA1 for Nox1; p47phox/p67phox/Rac for Nox2; NOXO1 for Nox3; no subunits for Nox4 or Nox5) resulted in marked production of reactive oxygen. Small interfering RNAs decreased endogenous p22phox expression and inhibited reactive oxygen generation from Nox1, Nox2, Nox3 and Nox4, but not Nox5. Truncated forms of p22phox that disrupted the PRR inhibited reactive oxygen generation from Nox1 and Nox2, but not from Nox4 and Nox5. Expression of p22phox (P156Q) inhibited NOXO1-stimulated Nox3-activity, but co-expression of NOXA1 overcame the inhibitory effect. The P157Q and P160Q mutations of p22phox showed selective inhibition of Nox2/p47phox/p67phox, and selectivity was specific for the organizing subunit (p47phox or NOXO1) rather than the Nox catalytic subunit. These studies stress the importance of p22phox for the function of Nox1, Nox2, Nox3 and Nox4, and emphasize the key role of the PRR for regulating Noxes whose activity is dependent upon p47phox or NOXO1.

Evidence over the last decades shows that reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and their reaction products, play crucial roles in diverse physiological processes including growth (1), regulation of vasodilatation (2), hormone synthesis (3), sperm-capacitation (4), fertilization (5), oxygen sensing (6), bone absorption (7), and the innate immune response (8-10). However, until the last several years, the enzymes responsible for generating these ROS were unknown, except for the phagocyte NADPH oxidase, a superoxide-generating enzyme whose catalytic moiety, flavocytochrome b558, is composed of a large catalytic subunit (gp91phox or Nox2) and a smaller subunit (p22phox). Beginning in 1999, novel homologues of gp91phox have been reported (11-17), and the Nox family now consists of 7
members in humans, including of 5 Noxes (the NAD(P)H oxidases Nox1, Nox2 (gp91phox), Nox3, Nox4, and Nox5) and two dual oxidases (Duox1 and Duox2). The latter contain a peroxidase homology domain in addition to the NADPH-oxidase domain. These homologues share a core structure consisting of six predicted transmembrane α-helices, which bind two heme groups, plus a C-terminal flavoprotein domain containing the conserved binding sites for NADPH and FAD. In addition to the core structure of Nox, Nox5 contains N-terminal extension containing calcium binding EF-hand motifs (for review, see refs. 18 and 19).

The molecular mechanisms underlying the activation of Nox2 have been studied extensively. Nox2 is complexed with a small subunit p22phox to form flavocytochrome b558 (9). Nox2 activation requires stimulus-induced assembly of the cytochrome b558 with the cytosolic regulatory proteins p47phox, p67phox, p40phox, and the small GTPase Rac (for review, see refs. 9, 20, and 21). In the resting state, p47phox and p67phox are located in the cytosol. Upon cell activation, e.g., exposure to opsonized bacteria, chemoattractants, or a protein kinase C activator phorbol 11-myristate 12-acetate (PMA), p47phox becomes hyperphosphorylated in the auto-inhibitory region (AIR), freeing its tandem SH3 domains to bind to a C-terminally located proline-rich region (PRR) of p22phox, and resulting in translocation from cytosol to the membrane-associated flavocytochrome b558 (22, 23). Following GTP binding, the small GTPase Rac translocates to the membrane (24). Both Rac and p47phox provide binding sites for p67phox, resulting in its association with Nox2 and activation of electron transfer from NADPH to FAD (25-28). Another cytosolic subunit p40phox was identified as a p67phox-binding protein and, while not essential, facilitates the stimulus-induced translocation of p47phox and p67phox to the membrane (29), resulting in increased Nox2 activity.

Recently, homologues of p47phox and p67phox were reported by several groups and have been named Nox organizer 1 (NOXO1) and Nox activator 1 (NOXA1), respectively (30-33). Several biochemical studies indicate that like gp91phox, Nox1 activity is also governed by regulatory subunits, in this case NOXO1 and NOXA1, since Nox1-dependent reactive oxygen generation required co-expression of both NOXO1 and NOXA1 (30-33). In addition, that of NOXO1 and Nox1 are coordinately expressed in the gastrointestinal tract, consistent with NOXO1 as a physiological regulatory subunit for Nox1 (31,34,35).

Other Noxes show both subunit-dependent and subunit-independent regulation. Human Nox3 requires regulatory subunits, but its regulation is less discriminating than that of Nox1. Nox3 can be activated by NOXO1 alone, or by the combination of p47phox and p67phox in a PMA-dependent manner (36). Mouse Nox3 shows similar regulation, except that it requires NOXA1 in combination with NOXO1 (37). On the other hand, Nox4 is constitutively active (14) and it is not activated by these subunits (38). Recent studies have demonstrated that insulin receptor- or toll-like receptor 4-signaling pathway increase Nox4-derived ROS generation (39, 40), but the mechanism of this regulation is not known. Nox5-dependent ROS generation is activated by calcium, consistent with its N-terminal calcium-binding domain (16, 41). Similarly, a preparation of Duox1 and Duox2 partially purified from thyroid is also activated by calcium (42). Roles for additional subunits for calcium-dependent Noxes have not been reported.

Despite an increasing number of studies documenting the roles of regulatory subunits in Nox regulation, the role of p22phox in Nox family members other than Nox2 remains relatively unexplored. Activity studies have been hampered by the near universal expression of p22phox in experimental cell systems. We thus used siRNA in the present studies to inhibit expression of endogenous p22phox and to determine the requirement of Nox1, Nox2, Nox3, Nox4, and Nox5 for this subunit. We find that Nox1, Nox2, Nox3, and...
Nox4 require p22phox, but the calcium-dependent enzyme Nox5 does not. In addition, for each of these systems, we have investigated the importance of the PRR of p22phox in regulating ROS generation. Unexpectedly, we have found that forms of p22phox mutated in this region function as dominant inhibitors for Nox1, Nox2, and Nox3 activities, but not for Nox4 or Nox5. In addition, specific mutations can discriminate between p47phox-dependent regulation and NOXO1-dependent regulation. The present studies implicate both regulatory subunit binding-dependent and -independent functions of p22phox in regulating ROS production by Nox enzymes.

**Experimental Procedures**

**Cells and Reagents.** - Human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s modified Eagles medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (36). Chicken anti-human NOXO1 and NOXA1 antibodies were previously described (36). Monoclonal antibodies 44.1 and NS2 against human p22phox were kindly provided by Dr. A. Jesaitis (Montana State University, Bozeman, MT). Polyclonal antibody R3179 was prepared against human neutrophil p22phox and was shown previously to be specific for p22phox (43). Monoclonal antibody E39.1, raised against a synthetic peptide representing amino acid residues 235-248 of human Nox1, was previously described (36). Reverse Transcription-PCR - Total RNA was isolated from HEK293 cells with TRIzol (Invitrogen). cDNA was synthesized either with or without MuLV reverse transcriptase (Applied Biosystems). Polymerase chain reaction (PCR) was performed with GeneAmp PCR system (Applied Biosystems) using the following primer sets: p22phox, 5'-ATGGGGCAGATCGAGTGGGCCATGT-3' and 5'-GTAGATGCCGCTCGCAATGGCCAG-3'; glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-TCATGACCACAGTCCATGCCATCACT-3' and 5'-GCTATGCCCCCCAGCTGAGCGGCG-3'; amplification was performed in both strands using ABI model 377 sequencer and was identified as Nox5α (GenBank accession No. AF317889).

**Reverse Transcription-PCR.** - Total RNA was isolated from HEK293 cells with TRIZol (Invitrogen). cDNA was synthesized either with or without MuLV reverse transcriptase (Applied Biosystems). Polymerase chain reaction (PCR) was performed with GeneAmp PCR system (Applied Biosystems) using the following primer sets: p22phox, 5'-ATGGGGCAGATCGAGTGGGCCATGT-3' and 5'-GTAGATGCCGCTCGCAATGGCCAG-3'; glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-TCATGACCACAGTCCATGCCATCACT-3' and 5'-GCTATGCCCCCCAGCTGAGCGGCG-3'. Amplified cDNA fragments were ligated into a pCR4-TOPO vector (Invitrogen). Each product was confirmed to be the
corresponding cDNA fragment using ABI model 377 sequencer.

**Preparation of shRNAs against Human p22phox** - DNA oligonucleotides encoding short hairpin RNA (shRNA) sequences with loop sequence (TTCAAGAGA) were subcloned into the pSuper-neo vector (OligoEngine) according to the manufacturer’s instructions. Specific shRNA sequences were selected on the basis of human p22phox cDNA sequence (GenBank™ Accession No. NM_000101) as follows: 5’-AACATGACCGCGTGTTGAAG-3’ for shRNA-p22-N, 5’-AATAGTCCTGAGCAGCCCCGC-3’ for scrambled shRNA-p22-N, 5’-AAGGAAATTACATATGGCGGC-3’ for shRNA-p22-M, 5’-AACGGCATCTACCTACTGGCGGC-3’ for shRNA-p22-C, and 5’-AATGGCTGGTCAACGGTACCC-3’ for scrambled shRNA-p22-C. All the constructs were verified by sequencing.

**Generation of Truncation and Point Mutation of p22phox, GFP-fused Nox1, and Myc-tagged Nox4** - We amplified the DNA fragments that encode native p22phox and truncated forms of p22phox (amino acid residues 1-149, 1-155, and 1-172) by PCR using sense primer (5’-AAAAAGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-5’) and antisense primers as follows: full length p22phox, 5’-AAAAGGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-3’; p22phox (residues 1-149), 5’-AAAAAGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-3’; p22phox (residues 1-155), 5’-AAAAGGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-3’; and p22phox (residues 1-172), 5’-AAAAGGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-3’. These primers contained BamH1 and EcoR1 sites. After digestion by each enzyme, the cDNA fragments were subcloned into the pcDNA3.1 vector. Using a similar strategy, p22phox (P159Q), p22phox (P157Q), p22phox (P156Q), and full-length p22phox DNAs were subcloned into the pcDNA3.1 (Invitrogen).

To generate cDNA encodes N-terminal GFP-fused protein of Nox1, human Nox1 cDNA was amplified using sense primer (AAAAAGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-5’) and antisense primer (AAAAAGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-3’). This sense primer has been designed to delete the initial methionine of native Nox1. The PCR product contained SacI (italic) and SalI (underline) sites. For C-terminally myc-tagged Nox4 protein, human Nox4 cDNA was amplified using sense primer (AAAAAGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-5’) and antisense primer (AAAAAGATCCGGTATTCCAGGGAGATCGAGTGGGCGCCA-3’). These primers contained EcoR1 (italic) and SalI (underline) sites. After digestion by each enzyme, the cDNA fragments including Nox1 and Nox4 cDNAs were subcloned into the pEGFP-C3 vector (BD Biosciences) and the pcDNA3.1 vector, respectively. All constructs were verified by sequencing.

**Transient Transfections** - HEK293 cells were grown 24 hrs in 6-well-plate and allowed to reach to 50% confluence in 2 ml culture medium. Cells were transfected with vectors carrying Nox1 through Nox5, p67phox, p47phox, NOXO1, NOXA1, full-length p22phox, and...
mutated or truncated p22phox cDNAs alone or in the indicated combinations using the transfection reagent FuGENE 6 (Roche Diagnostics) according to manufacturer’s instructions. After 48 hrs, or 72 hrs when co-transfected with shRNA vectors, cells were washed twice with cold Hank’s balanced salt solution (HBSS) and were removed from the well, after which cells were harvested by centrifuging at 500 x g for 5 min and re-suspending in HBSS.

**Measurement of ROS** - ROS was measured using luminol luminescence as previously described (33, 36). Cells (4 x 10^4) in HBSS with calcium and magnesium were mixed with 200 µM luminol plus 0.32 units of horseradish peroxidase in a 200 µl total volume in each well of a 96-well plate. Luminescence was quantified using FluoStar™ luminometer (BMG Labtech), recording data every minute for 2 hrs. Luminescence increased during early time points, peaking at 30 min, and this value was reported herein. For the measurement of Nox2 activation, cells were pre-incubated with 200 nM PMA for 5 min at 37°C. For Nox5 activation, cells were stimulated with 1 µM ionomycin or the same amount of vehicle at 10 min after beginning data collection, and immediately achieved the maximum rate, which was reported at the 12 minute time point.

**Western Blot Analysis** - Cells were lysed in buffer containing 6 M urea, 25% glycerol, 0.1% SDS, 0.5% sodium deoxycholic acid, 1% Nonidet P40 substitute (Sigma), 150 mM NaCl, 50 mM Tris/HCl (pH 7.8), protease inhibitor cocktail (Complete™, Roche Diagnostics), 1 mM phenylmethylsulfonyl fluoride, and 100 µM diisopropyl fluorophosphate. Cell lysates were then incubated with Laemmeli sample buffer (BioRad) containing 5% 2-mercaptoethanol for 30 min at room temperature. Extracted protein from approximately 2 x 10^5 cell equivalents was resolved by 15% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Following blocking with nonfat dry milk (4%), proteins were probed using their respective antibodies. Visualization was carried out with an enhanced chemiluminescent substrate kit (Pierce) using horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG, purchased from BioRad, or against chicken IgG from Sigma.

**Immunostaining** - Slides were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. After washing with PBS, tissue sections were permeabilized using 0.1% triton X-100 at 4°C for 5 min and were blocked with 1% goat serum at room temperature for 1 h. Slides were incubated with primary antibodies (monoclonal anti-Nox1 antibody E39.1 and rabbit polyclonal anti-p22phox antibody R3179 for colon, or rabbit polyclonal anti-Nox4 antibody and monoclonal anti-p22phox antibody 44.1 for kidney) for 2 h. After washing for 30 min, slides were incubated with the appropriate secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 goat anti-rabbit IgG for the colon or Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG for the kidney) for 1 h. Nuclei were stained with ToPro3 (Molecular Probes) for 20 min at room temperature and slides were washed for 15 min. Tissues were mounted in Vectashield (Vector Laboratories) and fluorescence images were scanned with a confocal laser scanning microscope (LSM 510 Meta, ZEISS). Controls were incubated with secondary antibodies only.

**Phylogenic analysis of human Nox family** - Multiple sequence alignment and phylogenic analysis were carried out with the programs Clustal W (http://www.ebi.ac.uk/clustalw/) and TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treewv.html). To perform alignment, we used human Nox1 (GenBank™ accession No. AF127763), human Nox2 (NM_000397), human Nox3 (AF190122), human Nox4 (AF254621), human Nox5 (AF353088), Drosophila melanogaster Nox (FlyBase CG3896), and Arabidopsis thaliana Nox (rbohA, GenBank™ accession No. AB015475) that was defined as an outgroup.
Results

Effects of \(p22^{phox}\) Expression on Activation of Nox Enzymes - Cellular ROS-generating systems that utilize Nox enzymes and regulatory proteins co-expressed in HEK293 cells have been reported previously (30-33,36). For example, co-expression of Nox2, \(p67^{phox}\), \(p47^{phox}\), and V12Rac1 resulted in high rates of PMA-dependent reactive oxygen generation. However, the reconstituted systems do not require ectopic expression of \(p22^{phox}\). Fig. 1A demonstrates that untransfected HEK293 cells express \(p22^{phox}\) message, suggesting that endogenous expression of \(p22^{phox}\) might support the activation of Nox enzymes without the need for additional overexpressed protein. In addition, we investigated a number of other cell types to try to find a cell line lacking \(p22^{phox}\) that was capable of reconstituting Nox-dependent activities, and all but one contained endogenous \(p22^{phox}\). We confirmed that human pulmonary carcinoma H292 cells (ATCC), which have been reported to lack endogenous \(p22^{phox}\) (Dr. Ulla Knaus, personal communication) fail to express \(p22^{phox}\), but for unknown reasons, H292 cells transfected with an expression plasmid encoding this subunit, also failed to express ectopic \(p22^{phox}\) (data not shown). In these cells, either with or without co-transfection with \(p22^{phox}\), only Nox5 was active, and no activity was seen with Nox1, Nox2, Nox3, and Nox4 when appropriate subunits were co-expressed (supplemental data, Fig. S1). Therefore, Nox5 does not require \(p22^{phox}\) for activity, but the H292 cell type did not appear to be suitable for studies of Nox1 through Nox4.

We therefore designed shRNA vectors against human \(p22^{phox}\) mRNA, which encode short interfering RNAs to suppress gene expression (44). Western blot analysis showed that transfection of shRNA-p22-N and shRNA-p22-C (but not shRNA-p22-M, scrambled shRNA-p22-N, scrambled shRNA-p22-C, and the empty vector) strongly suppressed the expression of \(p22^{phox}\) protein (Fig. 1B). Nox1-dependent activity correlated with \(p22^{phox}\) expression; shRNA-p22-N and shRNA-p22-C, but not empty vector, strongly suppressed the Nox1-derived ROS production (Fig. 2A). As expected from the well-established role of \(p22^{phox}\) in phagocyte ROS generation, the silencing of \(p22^{phox}\) also abrogated Nox2 activity (Fig. 2B). In contrast, scrambled shRNA-p22-N, scrambled shRNA-p22-C, and shRNA-p22-M did not affect Nox1- and Nox2-dependent ROS generation (supplemental data, Fig. S2). Transfection of \(p22^{phox}\)-shRNA vectors did not affect protein levels of co-expressed subunits, NOXO1, NOXA1, \(p47^{phox}\), and \(p67^{phox}\) (data not shown).

Furthermore, Fig. 2C shows that NOXO1-supported Nox3 activity also required \(p22^{phox}\). Similarly, \(p22^{phox}\) silencing also diminished Nox3 activities regulated by NOXA1 alone, NOXO1/NOXA1, \(p47^{phox}\) alone, or \(p47^{phox}\)/\(p67^{phox}\) (supplemental data, Fig. S3). These data suggest that \(p22^{phox}\) protein is broadly involved in Nox3-dependent ROS generation. In contrast to Nox1 through Nox3, Nox4-dependent ROS-generating activity does not require regulatory subunits, but \(p22^{phox}\) expression was still needed for Nox4 activity (Fig. 2D). As shown in Fig. S2, scrambled shRNA-p22-N and -C as well as shRNA-p22-M did not change the rates of Nox3- and Nox4-dependent ROS generation.

Banfi et al. showed that Nox5 activity is stimulated by calcium ion/ionomycin (41). In the present studies, low level basal Nox5-dependent ROS generation was seen without the addition of calcium ionophore, and this activity was markedly increased by ionomycin (Fig. 2E, hatched bars). In contrast to the other Nox enzymes, the silencing of \(p22^{phox}\) did not affect either the spontaneous or the ionomycin-triggered ROS generation by Nox5 (Fig. 2E).

Nox2 (gp91\(^{phox}\)) protein has previously been shown to be stabilized by \(p22^{phox}\), since in a form of chronic granulomatous disease lacking \(p22^{phox}\), Nox2 expression is also impaired (45). Therefore, the decreased activity seen above when expression of \(p22^{phox}\) is impaired might be due to decreased expression of Nox subunits. To investigate whether silencing of \(p22^{phox}\) affects expression of Nox1 protein, we examined the expression of N-terminal GFP-fused Nox1 vector. A GFP
fusion protein was utilized, since examination of several antibodies against Nox1 failed to reveal Nox1 expression by Western blotting. The N-terminal fusion was selected, since fusion with GFP at the N-terminus resulted in full activity (supplemental data, Fig. S4), whereas GFP fused at the C-terminus resulted in complete absence of Nox1 activity (33). When GFP-Nox1 was co-expressed with NOXO1 and NOXA1 in HEK293 cells, shRNA-p22-N and -C vectors significantly suppressed ROS generation by GFP-Nox1 (Fig. S4). The knockdown of p22\textsubscript{phox} resulted in an approximately 50% decrease in GFP-Nox1 expression (Fig. 3A). However, for shRNA-p22-N, the decreased expression of Nox1 was insufficient to account for the nearly complete inhibition of ROS generation by this vector, suggesting that Nox1-dependent ROS generation utilizes p22\textsubscript{phox} not only for stabilization, but also for other functions. Nox4 fused at the C-terminus with the myc epitope retained approximately 15% of the ROS-generating activity compared with untagged Nox4 protein (supplemental data, Fig. S4). Silencing of p22\textsubscript{phox} completely suppressed ROS generation by myc-tagged Nox4 (Fig. S4). However, under these conditions, the expression of Nox4 protein was not significantly affected by silencing of p22\textsubscript{phox} (Fig. 3B). Hence, Nox4 also requires p22\textsubscript{phox} for functions other than stabilization of the Nox4 catalytic subunit.

**Dominant Inhibitor Effects of the C-terminal Truncations of p22\textsubscript{phox} on Activation of Nox Enzymes.** For activation of the phagocyte NADPH oxidase containing Nox2, the p22\textsubscript{phox} subunit plays two critical roles: 1) formation of a stabilizing complex with Nox2 and; 2) mediation of the assembly between flavocytochrome b\textsubscript{558} and cytosolic regulatory subunits (9, 20, and 21). Based on this idea, we hypothesized that a mutant of p22\textsubscript{phox} lacking an intact PRR would still associate with the Nox subunit, but would fail to mediate interactions with regulatory subunits. As such, these mutant forms of p22\textsubscript{phox} could compete with wild type p22\textsubscript{phox} and would therefore function as dominant inhibitors for Noxes that require an organizer protein subunit such as p47\textsubscript{phox} or NOXO1. Binding of p47\textsubscript{phox} requires direct interaction between the PRR of p22\textsubscript{phox} (amino acids 151-160) and the tandem SH3 domains of p47\textsubscript{phox} (28). Consistent with this hypothesis, the C-terminal truncations p22\textsubscript{phox}(1-149) and p22\textsubscript{phox}(1-155) that disrupt the PRR, inhibited ROS generation by Nox1, Nox2, and Nox3 (Figs. 4, A, B, and C, respectively). These inhibitory effects were not observed using either full-length p22\textsubscript{phox} or p22\textsubscript{phox}(1-172) in which the PRR is intact (Figs. 4, A-C). As seen in Fig. 2, Nox4 activity requires the p22\textsubscript{phox} subunit, but truncated forms of p22\textsubscript{phox} failed to inhibit Nox4-dependent activity (Fig. 4D). Similarly, neither full-length nor truncated forms of p22\textsubscript{phox} affected the calcium-dependent or -independent activities of Nox5 (Fig. 4E).

**Dominant Inhibitor Effects of the Point Mutated p22\textsubscript{phox} (P156Q) on the Activity of Nox Enzymes.** The missense mutation p22\textsubscript{phox}(P156Q) is causally linked to chronic granulomatous disease, a condition in that is characterized by impaired ability of the neutrophil NADPH oxidase (Nox2) to produced microbicidal ROS (46). Previous biochemical studies demonstrated that p22\textsubscript{phox}(P156Q) fails to bind to p47\textsubscript{phox} (28,46,47). To examine whether mutation of the PRR would cause p22\textsubscript{phox} to function as a dominant inhibitor, experiments similar those in Fig. 4 were performed using p22\textsubscript{phox}(P156Q). As with truncated forms, co-expression of p22\textsubscript{phox}(P156Q) markedly suppressed Nox1-dependent ROS generation in a dose-dependent manner (Fig. 5A), with greater than 95% inhibition at a vector concentration of 0.25 µg/ml. Similarly, when this amount of p22\textsubscript{phox}(P156Q) was expressed, Nox2 activity was also completely inhibited (Fig. 5B). Like Nox1 and Nox2, p22\textsubscript{phox}(P156Q) abrogated NOXO1-dependent Nox3 activation (Fig. 5C). Consistent with the results shown in Fig. 4, p22\textsubscript{phox} (P156Q) did not influence Nox4 and Nox5 activities (Figs. 5, D and E, respectively).

**Effects of Point Mutation p22\textsubscript{phox} (P156Q) on Nox3 Activities regulated by “Activator” Subunits.** Unlike Nox1 and Nox2, Nox3...
activity can be modestly activated by the “activator” subunit NOXA1 or p67\textsubscript{phox} by itself in the absence of an organizer subunit (36). Unlike NOXO1-dependent activation, NOXA1-dependent Nox3 activity was not affected by the p22\textsubscript{phox} mutant (Fig. 6A). In the presence of NOXO1 plus NOXA1, activity was significantly higher, about the same as the activity supported by NOXO1 alone. Remarkably, co-expression of NOXA1 along with NOXO1 completely rescued the dominant inhibitor effect of p22\textsubscript{phox} (P156Q) towards Nox3 activity (Fig. 6B). Similarly, Nox3 activity regulated by p67\textsubscript{phox} alone or in combination with p67\textsubscript{phox} was not affected by p22\textsubscript{phox} (P156Q) (Figs. 6, C and D). The explanation for this phenomenon is not entirely clear and will require additional investigation. Nevertheless, results indicate that the activator protein (NOXA1 or p67\textsubscript{phox}), when complexed to an organizer protein, bypasses the requirement for interaction with the PRR of p22\textsubscript{phox}, perhaps by allowing the activator protein to bind directly to the Nox3 protein.

**Differential Specificities of Point Mutations in the Proline-Rich Region of p22\textsubscript{phox} for Inhibiting Nox1 versus Nox2.** - The PRR of p22\textsubscript{phox} possesses prolines, at position 151, 152, 155, 156, 157, 159, and 160. To confirm that dysfunction of the PRR structure caused the dominant inhibitor effects towards Nox1 and Nox2, we generated three additional amino acid substitutions in this region (P157Q, P159Q, or P160Q). Among them, p22\textsubscript{phox}(P157Q) and p22\textsubscript{phox}(P159Q) inhibited Nox1 activity, but their effects were less than p22\textsubscript{phox}(P156Q) (Fig. 7A). In contrast, p22\textsubscript{phox}(P160Q) failed to inhibit significantly (Fig. 7A). Interestingly, when we examined their effects on Nox2, two of these point mutations showed different specificity compared with that for Nox1 inhibition; p22\textsubscript{phox}(P157Q) inhibited Nox2 activity much more effectively than Nox1 activity, and p22\textsubscript{phox}(P160Q) functioned as a moderately strong inhibitor of Nox2 but had little effect on Nox1 (Fig. 7B).

To examine whether the differences in specificity were dictated by the catalytic subunits, or by the co-expressed organizer proteins (p47\textsubscript{phox} or NOXO1), we tested heterologous combinations in which NOXO1 and p47\textsubscript{phox} were substituted for one another in the Nox1 and Nox2 systems, i.e. Nox1 (Nox1, p47\textsubscript{phox}, and NOXO1) and Nox2 (Nox2, NOXO1, p67\textsubscript{phox}, and V12Rac1). Inspection of Figs. 7, C and D, reveals that the inhibitor specificity of different p22\textsubscript{phox} mutants was independent of the Nox subunit, and was dictated solely by which organizer subunit was expressed in the assay cells.

**Effect of Point Mutation and Truncation of p22\textsubscript{phox} on Nox1 Expression.** - To test whether any of the inhibitory effect of p22\textsubscript{phox} mutants might be attributed to expression of Nox1 protein, we examined the expression of N-terminally GFP-fused Nox1. As with the native Nox1, p22\textsubscript{phox} truncations (1-149 or 1-155) and mutations (P156Q, P157Q, or P159Q) also markedly inhibited the activity of the GFP-Nox1 protein (Fig. 8). Protein expression levels of GFP-Nox1 and subunits (NOXO1 and NOXA1) were not affected by co-expression of either wild type or mutant forms of p22\textsubscript{phox} (Fig. 8). Thus, inhibitory effects of these subunits are not due to altered expression of Nox subunits.

**Colocalization of p22\textsubscript{phox} and Nox1 or Nox4 in human tissues.** - According to previous reports, human Nox1 and Nox4 mRNAs are predominantly expressed in the colon and kidney, respectively (11, 15). To investigate whether p22\textsubscript{phox} colocalized with endogenous Nox1 and Nox4 in vivo, we performed immunofluorescence analysis of the human colon epithelium and the renal cortex. Nox1 was predominantly expressed on the apical side of human colon epithelial cells. p22\textsubscript{phox} was expressed somewhat more broadly in epithelium, but was highly expressed with the same subcellular location as Nox1, colocalizing at the surface of the epithelial cells (Figs. 9, A and B). Strong expression of Nox4 and p22\textsubscript{phox} was observed in the epithelial cells of kidney tubules (Figs. 9, D and E) where colocalization was seen in membranes and submembranous vesicle-like structures. Thus, coexpression results are
consistent with a role for p22phox as a physiological partner of Nox1 and Nox4.

Discussion

Silencing of endogenous p22phox expression demonstrated that p22phox is required for the activities of Nox1 through Nox4 (Fig. 2), but not the calcium-dependent Nox5. This is consistent with the idea, first developed from co-isolation of Nox2 and p22phox, that p22phox forms a functional complex with some Nox catalytic subunits, and with recent biochemical data that used fluorescence resonance energy transfer and co-immunoprecipitation to demonstrate that p22phox interacts with GFP fusion proteins of Nox1 and Nox4 (38). Recently, Ueno et al. (48) also demonstrated that the myc-tagged Nox3 binds to p22phox using co-immunoprecipitation analysis.

As shown herein, human Nox5 activity was calcium-stimulated, but did not require p22phox. Nox5 contains a unique N-terminal extension including four EF-hand motifs and single proline/arginine-rich region that is predicted to be exposed on the cytosolic side of the membrane C-terminal to the calcium-binding region (13, 16). It has been suggested that this N-terminal extension of Nox5 might function in a manner similar to p22phox. Nevertheless, it remains unknown whether Nox5 requires another endogenously expressed regulatory subunit(s). We have identified the Drosophila melanogaster Nox5 homologue gene DmNox (CG3896 on FlyBase, see refs. 49, 50), and several Nox5 homologues (termed the rboh proteins) have been reported in plants including Arabidopsis thaliana (51) and tobacco (52). According to the predicted amino acid sequences, DmNox and rboh possess conserved NADPH-oxidase domains and N-terminal extension containing EF-hand motifs. On the other hand, extensive BLAST genome search has failed to identify a p22phox homologue in fly or plants. To help to interpret the relationships among human Nox family and the requirement of p22phox for ROS-producing activity, a phylogenetic tree of human Nox1 through Nox5, DmNox, and plant Nox (rbohA) is shown (Fig. 10). The requirement of p22phox and for the PRR is shown to the right side of the dendrogram. According to the dendrogram, human Nox5 is closer to fly Nox (DmNox) and plant Nox (rboh) than other human Noxes. Thus, the present studies provide a rationale for why a p22phox homologue is not present in these species.

Earlier studies have noted partial activation of Nox1 and Nox4 by ectopic expression of p22phox. Takeya et al. showed that co-expression of p22phox together with Nox1 and its other regulatory subunits (Nox1, NOXO1, and NOXA1) stimulates Nox1-dependent superoxide generation (32), but it was not clear whether p22phox was absolutely required for activity nor whether its effect was due to stabilization of Nox1 versus regulation of its activity. Similarly, co-transfection of rat p22phox was reported to cause a ~3-fold increase in ROS generation in HEK293 expressing rat Nox4-transfected cells compared with Nox4 alone (38). However, in our experiments expressing human proteins, excess expression of wild type p22phox did not further increase human Nox-enzyme-activities (Fig. 4), possibly due to different sub-lines of these cells in use in different labs and/or the use of rodent versus human proteins. The failure of exogenous p22phox expression to affect Nox-dependent activities indicates that in the HEK293 cells used in our laboratory, there is sufficient expression of endogenous p22phox to fully support the activities of these Nox enzymes. The use of shRNA to suppress p22phox expression demonstrates for the first time a requirement of Nox3 for p22phox and the independence of the calcium-regulated Nox5 of p22phox. In addition, these approaches demonstrate that Nox4- and Nox1-dependent activities are absolutely dependent upon p22phox since there is little or no activity in the absence of this subunit.

The requirement for p22phox may arise either from stabilization of the catalytic Nox subunit by the smaller subunit, or from a functional role of p22phox related to catalysis or recruitment of regulatory subunits. The
former phenomenon has been well documented for Nox2, since in variants of chronic granulomatous disease, the absence of either the Nox2 or the p22phox subunit results in the absence of the other subunit, presumably due to protein degradation of the unpaired subunit (44). As seen in Fig. 3, Nox1 protein levels were decreased by silencing of p22phox, indicating that Nox1 is also stabilized to some extent by p22phox protein. However, significant Nox1 expression still occurs in the absence of p22phox and under these conditions, Nox1-dependent activity is nearly completely absent, pointing to additional roles of p22phox in Nox1-dependent ROS generation.

Regarding the latter, the role of the PRR of p22phox in the recruitment of p47phox via its bis-SH3 domain has been extensively studied including by structural methods, and the use of forms of p22phox mutated in the PRR has allowed us in the present studies to demonstrate that for Nox1, the PRR is essential for the activating function of p22phox. In contrast to Nox1 and Nox2, Nox4 expression was not affected when p22phox expression was blocked (Fig. 3) indicating that the effect of p22phox on Nox4 activity was not due to subunit stabilization. Thus, p22phox is playing some other regulatory role to maintain the activity of Nox4. Importantly, the present studies show that the PRR of p22phox does not participate in this regulation of Nox4. This is consistent with the absence of any requirement for NOXO1, NOXA1, p47phox or p67phox for Nox4-dependent ROS generation.

Herein, we demonstrate that p22phox(P156Q) is a strong inhibitor of both Nox1- and Nox2-dependent activities. In vitro binding assays using synthetic peptides corresponding to the PRR have demonstrated that wild type but not the peptide corresponding to the P156Q mutation binds to tandem SH3 domains of NOXO1 (32) like p47phox. Recently, Groemping et al. reported the X-ray crystal structure of the complex between p22phox and p47phox (53). According to details of the contacts, these interactions involve van der Waals interactions between Pro-156 in the PRR of p22phox and Trp-193 in the N-terminal SH3 domain of p47phox. Similarly, the residue Pro-157 of p22phox individually interacts with the Tyr-167, Pro-206, and Phe-209 in the N-terminal SH3 region, while Pro-160 of p22phox is stacked against the Phe-209 of p47phox. Interestingly, as shown in Fig. 7, p22phox(P157Q) and p22phox(P160Q) inhibited p47phox-supported ROS generation more effectively than that supported by NOXO1. These results are consistent with structural information described above because several residues of p47phox that are responsible for the interaction with p22phox are different in NOXO1. Specifically, Phe-209 of p47phox, which interacts with Pro-157 and Pro-206 of p22phox, is replaced by tyrosine in NOXO1. Similarly, Tyr-167 of p47phox, the residue that interacts with Pro-157 of p22phox, is changed to phenylalanine in NOXO1. This seems likely to explain the different inhibitory specificities when Pro-157 or Pro-160 in p22phox are mutated.

Mutants of p22phox may find utility as both tools for cell biology, and possibly as therapeutic agents. Inappropriate activation of Nox2 is associated with inflammatory conditions including shock lung, arthritis and damage to endothelium (54, 55), whereas overexpression of Nox1 may be implicated in cancer, atherosclerosis and hypertension (56, 57). Nox3 is implicated in otolith formation in the inner ear and hence plays a role in balance perception (58), while Nox4 has been shown to play a role in the insulin response (40). Thus, it is potentially useful to have therapeutic agents that can selectively inhibit one or a small number of Noxes with little or no effect on other Noxes. Currently, there are few if any selective inhibitors of Nox enzymes. Diphenylene iodonium has been used in a variety of Nox studies, but shows broad specificity for most or all Noxes. Unfortunately, this compound also inhibits a fairly broad range of flavoprotein dehydrogenases, and is therefore not very useful for cell biology studies or for as a therapeutic agent. Cell-internalized peptide corresponding to an intracellular loop of the transmembrane region of Nox2 or adenovirus
vector encoding antisense Nox2 partially inhibit angiotensin II-induced ROS generation in mouse vascular smooth muscle and the respiratory burst in human neutrophils (59, 60). Recently, fungal gliotoxin from *Aspergillus fumigatus* has been reported as a natural Nox2 inhibitor. Its mechanism of inhibition remains unclear, although it is known to abrogate phosphorylation of p47phox, preventing formation of an assembled, active Nox2 complex (61). In the present study, we demonstrated that the p22phox PRR mutants did not affect Nox4 activity in contrast to their potent effect on Nox1- and Nox2-dependent activities. This was despite the clear requirement of Nox4 ROS generation for p22phox. In addition, two of the mutants showed greater efficacy against p47phox-dependent rather than NOXO1-dependent processes, and are therefore expected to have greatest efficacy in Nox2-dependent ROS generation as is seen in phagocytes and selected other cell types including lung endothelium (54). Hence, if appropriate methods are developed for delivery or expression, these mutants might find use in the treatment of inflammatory conditions or other Nox2-dependent pathological conditions.

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Footnote
The abbreviations used are: ROS, reactive oxygen species; Nox, NADPH oxidase; Duox, dual oxidase; AIR, auto-inhibitory region; PRR, proline-rich region; SH3, Src homology 3; NOXO1, Nox organizer protein 1; NOXA1, Nox activator protein 1; PMA, phorbol 11-myristate 12-acetate; HEK, human embryonic kidney; PCR, polymerase chain reaction; RTase, reverse-transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA; GFP, green fluorescent protein; HBSS, Hank’s balanced salt solution.

Figure Legends

Fig. 1. Endogenous expression of p22phox in HEK293 cells. A. Total RNA isolated from HEK293 cells was subjected to the reverse-transcriptase (RTase) reaction with (+) or without (-) the enzyme as described in “Experimental Procedures”. cDNAs of p22phox and the reference GAPDH were amplified (25 cycles) using the specific primers. B. Whole cell protein from 72-hr transfected HEK293 cells along with 2 µg/ml of empty pSuper-neo vector (mock, lanes 3, 4, 11, and 12), shRNA-p22-N (lanes 5 and 6), shRNA-p22-M (lanes 7 and 8), shRNA-p22-C (lanes 9 and 10), scrambled shRNA-p22-N (lanes 13 and 14), scrambled shRNA-p22-C (lanes 15 and 16),...
or non-transfected cells (lanes 1 and 2) were resolved by 14% SDS-PAGE as described in “Experimental Procedures”. Western blot analysis was performed using mAb 44.1 against human p22phox. The results are representative of three separate experiments.

**Fig. 2. Effects of p22phox shRNAs on activation of Nox enzymes.** A-E, Combinations of Nox1 (each 0.1 µg/ml of Nox1, NOXO1, and NOXA1), Nox2 (each 0.1 µg/ml of Nox2, p47phox, and p67phox, and 0.25 µg/ml of V12Rac1), and Nox3 (each 0.1 µg/ml of Nox3 and NOXO1), and Nox4 (0.1 µg/ml) or Nox5 (0.1 µg/ml) alone were co-transfected in HEK293 cells along with pSuper-neo empty vector (mock), shRNA-p22-N, or shRNA-p22-C (each 2 µg/ml) for 72 hrs as described in “Experimental Procedures”. ROS generation was measured by luminol luminescence and indicated by RLU (relative luminescence units)/10⁴ cells, as described in “Experimental Procedures”. For Nox2 activation, PMA was added and preincubated at 37°C for 5 min before measurement of luminal activity. For Nox5 activation, cells were stimulated with 1 µM ionomycin (hatched bars) or same amount of vehicle (filled bars) after starting measurement at 10 min. Western blots represented under each bar indicate protein expression of p22phox (mAb 44.1) and the reference β-actin. Values are means ± SD (n = 6). Similar results were obtained in three separate experiments.

**Fig. 3. Effects of p22phox shRNAs on expression levels of Nox1 and Nox4 proteins.** A-B, Combination of GFP-fused Nox1 (each 0.2 µg/ml of GFP-Nox1, NOXO1, and NOXA1) and myc-tagged Nox4 (0.2 µg/ml) alone were co-transfected in HEK293 cells along with pSuper-neo empty vector (mock), shRNA-p22-N, or shRNA-p22-C (each 2 µg/ml) for 72 hrs as described in the legend to Fig. 2. Western blots indicate protein expression of GFP-fused Nox1 (anti-GFP Ab), myc-tagged Nox4 (anti-myc Ab), p22phox (mAb 44.1), and the reference β-actin. Positions for marker proteins are indicated in kDa and endogenous c-myc protein was indicated with an asterisk (B). Similar results were obtained in three separate experiments.

**Fig. 4. Negative effects of truncated forms of p22phox on activation of Nox enzymes.** A-E, Combinations of Nox1 through Nox3 as described in the legend to Fig. 2, or Nox4 and Nox5 alone were co-expressed in HEK293 cells along with the 0.25 µg/ml of empty pCMV-tag 5A vector (mock), p22phox, p22phox(1-149), p22phox(1-155), or p22phox(1-172). For Nox2 activation, PMA was added and preincubated for 5 min. For Nox5 activation, 1 µM ionomycin (hatched bars) or vehicle (filled bars) was added after starting measurement at 10 min. ROS generation was measured by luminol luminescence and indicated by RLU/10⁴ cells, as described in “Experimental Procedures”. Western blots represented under each bar indicate protein expression of p22phox (mAb NS-2) and the reference β-actin. Values are means ± SD (n = 6). Similar results were obtained in three separate experiments.

**Fig. 5. Negative Effects of point mutant p22phox (P156Q) on activation of Nox enzymes.** A-E, Combinations of Nox1 through Nox3 as described in the legend to Fig. 2, or Nox4 and Nox5 alone were co-expressed in HEK293 cells along with the indicated amount of empty pcDNA3 vector (mock), p22phox, or p22phox(P156Q). For Nox2 activation, PMA was added and preincubated for 5 min. For Nox5 activation, 1 µM ionomycin (hatched bars) or vehicle (filled bars) was added after starting measurement at 10 min. ROS generation was measured by luminol luminescence as described in “Experimental Procedures”, and indicated by RLU/10⁴ cells. Western blots represented under each bar indicate protein expression of p22phox (mAb 44.1) and the reference β-actin. Value are means ± SD (n = 6). Similar results were obtained in three separate experiments.
Fig. 6. Effects of point mutant p22\textsuperscript{phox} (P156Q) on activation of Nox3 in presence of “activator” subunit. A-D, HEK293 cells were transfected with different combinations of Nox3, NOXO1, NOXA1, p47\textsuperscript{phox}, and p67\textsuperscript{phox} (each 0.1 µg/ml) as indicated. In parallel, 0.25 µg/ml of pcDNA3 empty vector (mock), p22\textsuperscript{phox}, or p22\textsuperscript{phox} (P156Q) was also co-expressed in cells. ROS generation was measured by luminol luminescence and indicated by RLU/10\textsuperscript{4} cells as described in “Experimental Procedures”. Western blots represented under each bar indicate protein expression of p22\textsuperscript{phox} (mAb 44.1) and the reference β-actin. Value are means ± SD (n = 6). Similar results were obtained in three separate experiments.

Fig. 7. Effects of point mutant in the proline-rich-region of p22\textsuperscript{phox} on activation of Nox1, Nox2, and Nox4. A-E, Nox1 or Nox2 (each 0.1 µg/ml) was co-expressed in HEK293 cells together with 0.1 µg/ml of NOXO1, NOXA1, p47\textsuperscript{phox}, and p67\textsuperscript{phox}, or 0.25µg/ml of V12Rac1 in combinations as indicated. At the same time, 0.25 µg/ml of pcDNA3 empty vector (mock), p22\textsuperscript{phox}, p22\textsuperscript{phox} (P156Q), p22\textsuperscript{phox} (P157Q), p22\textsuperscript{phox} (P159Q), or p22\textsuperscript{phox} (P160Q), was co-expressed as indicated. Nox4 was also co-expressed in HEK293 cells along with 0.25 µg/ml of pcDNA3 empty vector (mock), p22\textsuperscript{phox}, p22\textsuperscript{phox} (P156Q), p22\textsuperscript{phox} (P157Q), p22\textsuperscript{phox} (P159Q), or p22\textsuperscript{phox} (P160Q). For Nox2 activation in presence of p47\textsuperscript{phox} or NOXO1, or for Nox1 activity in presence of p47\textsuperscript{phox}, PMA was added and preincubated for 5 min. ROS generation was measured by luminol luminescence and indicated by RLU/10\textsuperscript{4} cells, as described in “Experimental Procedures”. Western blots represented under each bar indicate protein expression of p22\textsuperscript{phox} (mAb 44.1) and the reference β-actin. Value are means ± SD (n = 6). Similar results were obtained in three separate experiments.

Fig. 8. Effects of p22\textsuperscript{phox} mutants on ROS generation and protein expression of GFP-Nox1. Each 0.2 µg/ml of GFP-tagged Nox1 (GFP-Nox1) or EGFP-C3 empty vector (GFP) was co-expressed in HEK293 cells along with NOXO1 and NOXA1 (each 0.2 µg/ml). In parallel, 0.25 µg/ml of pcDNA3 empty vector (mock), p22\textsuperscript{phox}, p22\textsuperscript{phox} (1-149), p22\textsuperscript{phox} (1-155), p22\textsuperscript{phox} (P156Q), p22\textsuperscript{phox} (P157Q), p22\textsuperscript{phox} (P159Q), or p22\textsuperscript{phox} (P160Q), was co-expressed in cells as indicated. ROS generation was measured by luminol luminescence and indicated by RLU/10\textsuperscript{4} cells, as described in “Experimental Procedures”. Western blots that represented under each bar indicate protein expression levels of GFP-Nox1, GFP, p22\textsuperscript{phox} (mAb NS-2), NOXO1, NOXA1, and the reference β-actin using corresponding antibodies. Positions for marker proteins are indicated in kDa. Values are mean ± SD (n = 6). Similar results were obtained in three separate experiments.

Fig. 9. Colocalization of p22\textsuperscript{phox} and Nox1 or Nox4 in human tissues. The human colon and kidney tissues were fixed and immunostained as in “Experimental Procedures”. A-C, anti-Nox1 monoclonal antibody (E39.1), anti-p22\textsuperscript{phox} polyclonal antibody (R3179), Alexa Fluor 488 goat anti-mouse IgG (green), and Alexa Fluor 555 goat anti-rabbit IgG (red) were used for detection of Nox1 and p22\textsuperscript{phox} in the colon. Arrows in A and B indicate the apical surface of epithelial cells. D-F, Anti-Nox4 rabbit polyclonal antibody and anti-p22\textsuperscript{phox} monoclonal antibody (44.1), Alexa Fluor 488 goat anti-rabbit IgG (green) and Alexa Fluor 555 goat anti-mouse IgG (red) were used for detection of Nox4 and p22\textsuperscript{phox} in the kidney. Arrows in D and E indicate kidney tubular epithelial cells. Nuclei were stained with ToPro3 (blue in A-F). For detection, tissues were viewed under a confocal laser scanning microscope. The same procedures were performed without primary antibody as negative controls (C and F). Magnifications are x 28 (A), x 120 (B and C), x 20 (D), x 232 (E), and x 63 (F). In each staining images, lower right panels showed merged images, and yellow or yellow-green color indicates co-localization. Similar results were obtained in three separate experiments.
Fig. 10. Relationships among human Nox family members and the requirement of p22phox for ROS-producing activity. Dendrogram was created from alignment of amino acid sequences of human Nox1 through Nox5, fruit fly Nox (DmNox), and *Arabidopsis thaliana* Nox (rbohA) that was defined as an outgroup. Evolutionary distances from a common ancestor sequence is represented as a bar meaning 0.1 nucleotide substitutions per site. The requirement for p22phox and for its proline-rich region for ROS-generating activity are indicated on the right side of dendrogram. For Nox3, the requirement for the proline-rich region of p22phox depends upon the combination of regulatory subunits that are present.
Kawahara et al. Fig. 7

A

Nox1/NOX01/NOXA1

RLU/10^6 cells

p22^{phox}

β-actin

mock

p22^{phox} (150)

p22^{phox} (300)

p22^{phox} (600)

p22^{phox} (1500)

B

Nox2/p47^{phox} 67^{phox} V12Rac1

RLU/10^6 cells

p22^{phox}

β-actin

mock

p22^{phox} (150)

p22^{phox} (300)

p22^{phox} (600)

p22^{phox} (1500)

C

Nox1/p47^{phox} NOX01

RLU/10^6 cells

p22^{phox}

β-actin

mock

p22^{phox} (150)

p22^{phox} (300)

p22^{phox} (600)

p22^{phox} (1500)

D

Nox2/NOX01/
p67^{phox} V12Rac1

RLU/10^6 cells

p22^{phox}

β-actin

mock

p22^{phox} (150)

p22^{phox} (300)

p22^{phox} (600)

p22^{phox} (1500)

E

Nox4

RLU/10^6 cells

p22^{phox}

β-actin

mock

p22^{phox} (150)

p22^{phox} (300)

p22^{phox} (600)

p22^{phox} (1500)
Figure legends

Supplemental Fig. S1. ROS generating activity of human pulmonary carcinoma H292 cells transfected with Nox enzymes. Combinations of Nox1 (each 0.5 µg/ml of Nox1, NOXO1, and NOXA1), Nox2 (each 0.5 µg/ml of Nox2, p47<sub>phox</sub>, and p67<sub>phox</sub>, and 1 µg/ml of V12Rac1), Nox3 (each 0.5 µg/ml of Nox3 and NOXO1), Nox4 (0.5 µg/ml), or Nox5 (0.5 µg/ml) alone or empty pCMV-5A vector (mock) were transfected in H292 cells (ATCC). For Nox2 activation, PMA was added and preincubated for 5 min. For Nox5 activation, cells were stimulated with 1 µM ionomycin (hatched bars) after starting measurement at 10 min. ROS generation was measured by luminol luminescence and the maximum rate was indicated by RLU/10<sup>4</sup> cells. Similar results are obtained in three separate experiments.

Supplemental Fig. S2. Effects of scrambled shRNA-p22-N, scrambled shRNA-p22-C, and shRNA-p22-M on enzymatic activities of Nox 1 through Nox5. Indicated combinations of Nox1 (each 0.1 µg/ml of Nox1, NOXO1, and NOXA1), Nox2 (each 0.1 µg/ml of Nox2, p47<sub>phox</sub>, and p67<sub>phox</sub>, and 0.25 µg/ml of V12Rac1), Nox3 (each 0.1 µg/ml of Nox3 and NOXO1), Nox4 (0.1 µg/ml), or Nox5 (0.1 µg/ml) alone were co-transfected along with pSuper-neo empty vector (mock), scrambled shRNA-p22-N, scrambled shRNA-p22-C, shRNA-p22-M (each 2 µg/ml) for 72 hrs. ROS generation was measured by luminol luminescence. Values are means ± SD (n = 4). Similar results are obtained in three separate experiments.

Supplemental Fig. S3. Effects of p22<sup>phox</sup> shRNAs on activation of Nox 3 in presence of “activator” subunit. Indicated combinations of Nox3 (each 0.1 µg/ml of Nox3/NOXA1, Nox3/NOXO1/NOXA1, Nox3/p67<sup>phox</sup>, or Nox3/p47<sup>phox</sup>/p67<sup>phox</sup>) were co-transfected along with pSuper-neo empty vector (mock), shRNA-p22-N, or shRNA-p22-C (each 2 µg/ml) for 72 hrs. ROS generation was measured by luminol luminescence and indicated by RLU/10<sup>4</sup> cells, as described in “Experimental Procedures”. Values are means ± SD (n = 6). Similar results are obtained in three separate experiments.

Supplemental Fig. S4. Effects of p22<sup>phox</sup> shRNAs on activation of GFP-fused Nox1 and myc-tagged Nox4. Indicated combinations of GFP-Nox1 (each 0.2 µg/ml of GFP-Nox1/NOXO1/NOXA1) or reference GFP (each 0.2 µg/ml of GFP/NOXO1/NOXA1) were co-transfected along with pSuper-neo empty vector (mock), shRNA-p22-N, or shRNA-p22-C (each 2 µg/ml) for 72 hrs. Myc-tagged Nox4 or reference untagged Nox4 (each 0.2 µg/ml) were also co-transfected along with pSuper-neo empty vector (mock), shRNA-p22-N, or shRNA-p22-C (each 2 µg/ml) for 72 hrs. ROS generation was measured by luminol luminescence and indicated by RLU/10<sup>4</sup> cells, as described in “Experimental Procedures”. Values are means ± SD (n = 6). Similar results are obtained in three separate experiments.
Kawahara et al. supplemental Fig. S1
Kawahara et al. supplemental Fig. S4

[Diagram showing RLU/10⁴ cells for GFP-NOX1/NOXA1 and myc-tagged Nox4/Nox4 under different conditions: mock, shRNA-p22 N, shRNA-p22 C, or mock.]
Point mutations in the proline-rich region of p22phox are dominant inhibitors of 
Nox1- and Nox2-dependent reactive oxygen generation
Tsukasa Kawahara, Darren Ritsick, Guangjie Cheng and J. David Lambeth

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