

# Nox5 Forms a Functional Oligomer Mediated by Self-Association of Its Dehydrogenase Domain

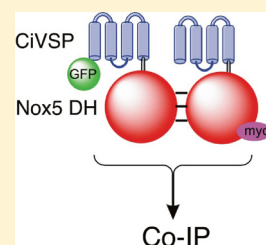
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**S** Supporting Information

**ABSTRACT:** Nox5 belongs to the calcium-regulated subfamily of NADPH oxidases (Nox). Like other calcium-regulated Noxes, Nox5 has an EF-hand-containing calcium-binding domain at its N-terminus, a transmembrane heme-containing region, and a C-terminal dehydrogenase (DH) domain that binds FAD and NADPH. While Nox1–4 require regulatory subunits, including p22<sup>phox</sup>, Nox5 activity does not depend on any subunits. We found that inactive point mutants and truncated forms of Nox5 (including the naturally expressed splice form, Nox5S) inhibit full-length Nox5, consistent with formation of a dominant negative complex. Oligomerization of full-length Nox5 was demonstrated using co-immunoprecipitation of coexpressed, differentially tagged forms of Nox5 and occurred in a manner independent of calcium ion. Several approaches were used to show that the DH domain mediates oligomerization: Nox5 could be isolated as a multimer when the calcium-binding domain and/or the N-terminal polybasic region (PBR-N) was deleted, but deletion of the DH domain eliminated oligomerization. Further, a chimera containing the transmembrane domain of *Ciona intestinalis* voltage sensor-containing phosphatase (CivVSP) fused to the Nox5 DH domain formed a co-immunoprecipitating complex with, and functioned as a dominant inhibitor of, full-length Nox5. Radiation inactivation of Nox5 overexpressed in HEK293 cells and endogenously expressed in human aortic smooth muscle cells indicated molecular masses of ~350 and ~300 kDa, respectively, consistent with a tetramer being the functionally active unit. Thus, Nox5 forms a catalytically active oligomer in the membrane that is mediated by its dehydrogenase domain. As a result of oligomerization, the short, calcium-independent splice form, Nox5S, may function as an endogenous inhibitor of calcium-stimulated ROS generation by full-length Nox5.



The family of human reactive oxygen species (ROS)-generating NADPH oxidases (Nox) consists of seven members, Nox1–5 and the dual oxidases Duox1 and Duox2.<sup>1</sup> Structural features and experimental data have been used to classify the Nox and Duox enzymes into two broad groups: those regulated by subunits including membrane-associated subunit p22<sup>phox</sup> (Nox1–4) and those that are regulated by calcium ion (Nox5 and Duox1 and -2).<sup>2–4</sup> In the former subgroup, Nox forms a tightly associated, mutually stabilizing heterodimer with p22<sup>phox</sup>. Nox1–3 enzymes require additional regulatory subunits such as p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, NOXO1, NOXA1, and the small GTPase Rac.<sup>5</sup> The calcium-regulated subgroup possesses a calcium regulatory domain that contains single or multiple EF-hand calcium-binding motif(s). While Duox1 and Duox2 require maturation factors DuoxA1 and DuoxA2, respectively, to facilitate their movement to the plasma membrane,<sup>6,7</sup> Nox5 apparently functions in a manner independent of subunits (ref 8 and vide infra).

Human Nox5 contains four EF-hand motifs in its calcium-binding domain, which is located at the N-terminus.<sup>9</sup> Rapid activation of Nox5 by calcium ion has been observed in Nox5-overexpressing HEK293 cells,<sup>8,9</sup> human aortic smooth muscle cells,<sup>10</sup> prostate cells,<sup>11</sup> and vascular endothelial cells.<sup>12,13</sup> In addition to calcium, other coactivating mechanisms cooperate by lowering the concentration of calcium needed to trigger activation: these include phorbol 12-myristate 13-acetate-dependent

phosphorylation of Nox5,<sup>14</sup> calmodulin,<sup>13,15</sup> and agonist-generated lipids, including phosphatidic acid and arachidonate (T. Kawahara and J. D. Lambeth, manuscript submitted for publication).

The physiological role of Nox5 has been extensively studied using *Drosophila melanogaster*, in which Nox5 (termed d-Nox) generates a ROS signal in smooth muscle to mediate hormone-induced smooth muscle contraction and egg laying.<sup>16</sup> In humans, Nox5 is expressed abundantly in the spleen, testis, and vascular tissue, cells of the gastrointestinal tract, and reproductive systems and is also seen in various cancers.<sup>17</sup> Nox5 has been lost in rodent genomes,<sup>2</sup> making it impossible to investigate its role in mouse knockout models. Results from in vitro studies in human cells indicate that Nox5-derived ROS enhances cell growth of prostate cancer cells,<sup>11</sup> hairy cell leukemia cells,<sup>18</sup> aortic smooth muscle cells,<sup>10</sup> and microvascular endothelial cells.<sup>12</sup> Recently, studies of human biopsy samples of coronary arteries demonstrated an increased level of expression of Nox5 in coronary artery disease, using immunoblotting, immunofluorescence, and quantitative polymerase chain reaction (PCR).<sup>19</sup>

These studies were undertaken on the basis of the observation (vide infra) that cotransfection of catalytically inactive point-mutated

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forms of several Nox isoforms along with the same wild-type isoform resulted in inhibition of wild-type enzyme activity, meeting the definition of a “dominant negative” genetic effect. There are at least two possible explanations for this type of phenomenon. One involves sequestration of a needed regulatory subunit or another component by the catalytically inactive enzyme, thereby removing a factor that is essential for the activity of the wild-type enzyme. A second class of explanations involves catalytically essential dimerization or higher-order oligomerization. According to the latter scenario, the inclusion of a catalytically inactive unit in the oligomer renders the whole inactive or partially active, for example, by creating a poorly active conformation of the whole. Because Nox5 does not require the participation of heterologous subunits, interpretation of the data is more straightforward than for other Nox isoforms, and this isoform was therefore selected for an in-depth investigation of the possibility of homo-oligomerization. A variety of methods were used to demonstrate oligomerization and to show that this interaction is essential for Nox5 function. In addition, several approaches were used to identify the region of Nox5 that mediates oligomerization.

## MATERIALS AND METHODS

**Materials.** Human embryonic kidney 293 (HEK293) cells and human aortic smooth muscle cells (HASMCs) were obtained from American Type Culture Collection (Manassas, VA) and Lonza (Walkersville, MD), respectively. Ionomycin, ferricytochrome *c*, superoxide dismutase (SOD), ethylenediamine-tetraacetic acid (EDTA), and a mouse monoclonal anti- $\beta$ -actin antibody were purchased from Sigma (St. Louis, MO). An anti-human Nox5 antibody was created in rabbits immunized with synthetic peptides corresponding to residues 491–506 and 577–588 of human Nox5 $\alpha$ . Anti-myc and -hexahistidine monoclonal antibodies, an anti-GFP monoclonal antibody, and an anti-GFP rabbit polyclonal antibody were purchased from Cell Signaling, Millipore, and Anaspec, respectively. Platelet-derived growth factor BB (PDGF-BB) was purchased from R&D Systems, Inc. Maltose dehydrogenase, alcohol dehydrogenase, and  $\beta$ -galactosidase were purchased from Sigma.

**Cell Culture.** HEK293 cells were grown for 24 h in six-well plates and allowed to reach 50% confluence in 2 mL of DMEM with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. HASMCs were maintained in smooth muscle growth medium {SmGM2 [smooth muscle basal medium (SmBM) with 5% FBS, 5 ng/mL insulin, 0.5 ng/mL EGF, 2 ng/mL FGF-B, and amphotericin B]}. Cells at passages 4–10 were used for the experiments.

**Constructs.** Expression vectors encoding wild-type, untagged Nox5 (subcloned in the PCMV-5A tag vector, Stratagene), C-terminally myc-tagged Nox5 (PCMV-5A tag vector), N-terminally hexahistidine-tagged Nox5 (PCMV-5A tag vector), and N-terminally GFP-tagged Nox5 (pEGFP-C3 vector, BD Bioscience) were previously described.<sup>20</sup> Point mutations were introduced by PCR-mediated mutagenesis as described previously.<sup>8</sup> Nox5-P567H was constructed by amplifying human Nox5 $\alpha$  cDNA (GenBank accession number AF353088) using primer set A (primer 1, GCATGGATCCACCATGAACACATCTGGA-GA; P567H-primer 2, CAGAATGGAAGCAAAGTGGGT-GATGCCGATGCC) and primer set B (P567H-primer 3, GGCATCGGCATCACCCACTTTGCTTCCATTCTG; primer 4, GCATAAGCTTCTAGAAATTCTCTTGAAAAATC-

TGAAGCCGAAC). Nucleotides encoding the mutated amino acid are underlined in primers 2 and 3. Primers 1 and 4 also introduce *Bam*HI (italics) and *Hind*III cleavage sites (underlined), respectively. Following a second round of PCR using primers 1 and 4 and digestion by each enzyme, the nucleotide fragments were subcloned into pCMV-tag 5A (Stratagene, La Jolla, CA). Point mutations V276R, L277R, L277A, P278L, P438A, D656A, and K658A of Nox5 were generated using a similar strategy. Truncated Nox5 (S59–F737) was constructed by amplification of the cDNA using a primer set (S59-primer 5, GCATAAGCTTTCCTTCTTG-CAGAGCGATTCTTT; primer 6, GCATGGATCCCTAGAA-ATTCTCTTGAAAAATCTGAAGCCGAAC). Primers 5 and 6 are designed to introduce *Bam*HI (italics) and *Hind*III cleavage sites (underlined), respectively. The nucleotide PCR products were subcloned into pEGFP-C3 (BD Bioscience, La Jolla, CA). Other truncations of M173–F737, A202–F737, A202–K429, and A202–A407 were made using a similar strategy.

The cDNA of the voltage sensor containing phosphatase from *Ciona intestinalis* (CiVSP, GenBank accession number AB18-3035) was kindly provided by Y. Okamura (Osaka University, Osaka, Japan). The N-terminus of the CiVSP (amino acids 1–239) containing the transmembrane region of the CiVSP protein was fused to the DH domain of Nox5 (amino acids 404–737 of Nox5 $\alpha$ ) to create the chimera termed “chimera CiVSP-N/Nox5-DH-C”. This chimera was made with either an N-terminal GFP tag or a C-terminal myc tag. These cDNAs were created by sequential PCRs followed by digestion and ligation into the appropriate vector. CiVSP-N/Nox5-DH-C-myc was constructed by amplification of the cDNA using primer sets: (1) CiVSP with *Bam*HI and Kozak sense (5'-TTTTGGATCCGCCACCATG-GAGGATTCGACGGTTCAG-3'), (2) CiVSP end at 239/Nox5 $\alpha$  begin at 404 sense (5'-GAATATTTTATTTCCCATCAA-CAAGAGAAGGCCATCGGACTG-3', with the CiVSP sequence underlined), (3) antisense of primer 2 (5'-CAGTCCGA-TGGCCTTCTCTTGTTGATGGGAATAAAATATTC-3'), and (4) Nox5 delete stop *Hind*III antisense (5'-TTTAAAGCT-TGAAATCTCTTGAAAAATCTGAAGCCG-3'). PCR A used primers 1 and 3 with CiVSP cDNA and primers 2 and 4 with Nox5 cDNA. PCR B used primers 1 and 4 with the two PCR products of PCR A. The final chimera product was digested with *Bam*HI and *Hind*III and ligated into pCMV tag 5A (Stratagene), which includes a C-terminal myc tag. To make CiVSP-N/Nox5-DH-C, the following primers were used: (5) CiVSP NotI sense (5'-TTTTCGCGCCGCATGGAGGGATTTCGACGGTTCAG-3'), (2 and 3) same as above, and (6) Nox5 stop *Hind*III antisense (5'-TTTAAAGCTTCTAGAAATCTCTTGAAAAATCTG-AAGCCG-3'). PCRs A and B were performed as described above. This chimera product was digested with *Not*I and *Hind*III and then ligated with the GFP-containing PCR product with a C-terminal *Not*I site and an N-terminal *Bam*HI site into pCMV tag 5A. The resulting cDNA is termed GFP-CiVSP-N/Nox5-DH-C. To construct GFP-CiVSP, CiVSP sense primer 7 with an N-terminal *Hind*III restriction enzyme site (5'-TTTAAAGCT-TATGGAGGGATTTCGACGGTTCAG-3') and an antisense primer 8 with a C-terminal *Bam*HI site (5'-TTTTGGATCCC-TAAATGTCTTCAGCATCTGAAAACG-3') were used for the PCR of full-length CiVSP. This product was digested with *Bam*HI and *Hind*III and ligated into pEGFP-C3, which encodes an N-terminal GFP tag. The CiVSP-myc construct was made with primer 1 listed above and antisense primer 9 with a *Hind*III

site and deletion of the stop codon (5'-TTTAAAGCTTAATG-TCTTCAGCATCTGAAAACG-3'). All sequences were confirmed by commercial DNA sequencing (Beckman Coulter Genomics).

**Immunoprecipitation.** HEK293 cells were plated in 10 cm dishes and allowed to grow for 24 h. Cells were then transfected with the indicated amount of each cDNA construct using Eugene-6 transfection reagents (Roche) according to the manufacturer's instructions. Cells were grown for 48 h and harvested in Hank's balanced salt solution (Gibco). Cell pellets were suspended in lysis buffer [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, and 150 mM NaCl] supplemented with EDTA-free protease inhibitor cocktail (Complete Mini, Roche), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 100  $\mu$ M diisopropyl fluorophosphate (DFP). Lysate mixtures were incubated on ice for 30 min and centrifuged. The supernatant containing proteins was incubated with the primary antibody overnight with rotation at 4 °C. A 50% protein-G Sepharose mixture was added to the lysates and rotated at 4 °C for 2 h. The protein-G Sepharose pellet containing immunoprecipitated proteins was washed three times with the Tris-HCl buffer described above. Laemmli sample buffer (Bio-Rad) containing 5% 2-mercaptoethanol was added to the immunoprecipitated proteins. Samples were resolved via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting.

**Measurement of ROS Generation in Intact Cells.** ROS was measured using luminol luminescence as previously described.<sup>8</sup> HEK293 cells were grown and transfected as described above. Forty-eight hours after transfection, cells were washed twice with Hank's balanced salt solution (HBSS) containing 1 mM calcium and 1 mM magnesium and harvested by centrifugation at 500g for 5 min. Cells ( $2 \times 10^5$ ) in HBSS with calcium and magnesium were mixed with 200  $\mu$ M luminol and 0.32 unit of horseradish peroxidase in a total volume of 200  $\mu$ L in each well of a 96-well plate. Luminescence was quantified using a FluoStar luminometer (BMG Labtech), recording data every 30 s for 1 h. Cells were treated with DMSO vehicle alone or with 1  $\mu$ M ionomycin in DMSO at 10 min after data collection had begun. The maximal luminescence was achieved within 1 min and is the value reported.

**Measurement of ROS Generation in Permeabilized Cells.** Superoxide in permeabilized HEK293 cells was measured using SOD-inhibitable cytochrome *c* reduction as previously described.<sup>20</sup> After pretreatment with 5  $\mu$ M thapsigargin to deplete intracellular calcium stores, cells were washed with calcium- and magnesium-free HBSS and were resuspended in phosphate-buffered saline (PBS, pH 7.2) containing a protease inhibitor mixture (Complete EDTA-free, Roche). To permeabilize cells, reduced streptolysin O (final concentration of 20 units/mL) was added to  $10^7$  cells in a volume of 10 mL and incubated for 10 min at 37 °C. Cells were chilled to 4 °C, centrifuged for 5 min at 1250g, and washed three times with PBS (pH 7.2). To measure the amount of superoxide, cells ( $\sim 5 \times 10^5$  cell equivalents) were suspended in calcium calibration solution [30 mM MOPS buffer (pH 7.2) containing 100 mM KCl, 1 mM CaEGTA, and 9 mM EGTA (Molecular Probes, Eugene, OR)], which results in a free calcium ion concentration of 17  $\mu$ M. Calcium-free buffer was the same, except that it lacked added calcium and contained 10 mM EGTA. After 5 min at room temperature, 1  $\mu$ L of the NADPH solution was added to give a final concentration of 100  $\mu$ M, with or without SOD (100 units/mL), and the rate of the absorbance increase at 550 nm was measured for 10 min using a Synergy HT spectrophotometer (Bio-Tek Instruments, Winooski, VT). Permeabilized cells were then lysed with 20 mM HEPES buffer

containing 1% Triton X-100, and the protein was quantified using the BCA protein assay reagent kit (Pierce, Rockford, IL). The rate of superoxide production was expressed as the amount of SOD-inhibitable cytochrome *c* reduced per minute per milligram of protein.

In some experiments, generation of H<sub>2</sub>O<sub>2</sub> by permeabilized HEK293 cells and HASMCs for radiation inactivation analysis was assessed by the fluorescence increase at 620 nm (excitation wavelength of 540 nm) because of Amplex Red oxidation in the presence of HRP, as described previously.<sup>21</sup> Permeabilized cells ( $\sim 5 \times 10^5$  cell equivalents) were kept frozen on dry ice during irradiation. For activity measurements, cells were thawed and suspended as described above in calcium calibration solution at 17  $\mu$ M free calcium ion or in calcium-free buffer. After 5 min at room temperature, hydrogen peroxide was measured with Amplex UltraRed with 200  $\mu$ L of a reaction buffer [30 mM MOPS (pH 7.2) containing 0.12 M NaCl, 3 mM KCl, 100  $\mu$ M Amplex Red, 200 milliunits/mL HRP, 20  $\mu$ M FAD, and 36  $\mu$ M NADPH]. The rate of fluorescence increase at 620 nm was measured at 25 °C for 10 min using a Synergy HT spectrofluorimeter (Bio-Tek Instruments). The rate of calcium-dependent H<sub>2</sub>O<sub>2</sub> production was expressed as the fluorescence increase at 620 nm per  $10^6$  cells minus that obtained in the calcium-free buffer.

**Immunoblotting.** Whole cell extract was obtained from cultured cells lysed in Laemmli sample buffer containing a protease inhibitor mixture (Complete), 100  $\mu$ M diisopropyl fluorophosphate, and 25 mM tris(2-carboxyethyl)phosphine (TCEP). Protein extracted from  $8 \times 10^4$  cell equivalents was resolved by 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) filters. After being blocked with bovine serum albumin (3%), proteins were probed using their respective primary antibodies and HRP-conjugated secondary antibodies against rabbit and mouse IgG (Bio-Rad Laboratories). Primary antibodies were used at dilutions of 1:1000 (anti-Nox5, -hexahistidine, -myc, and -GFP antibodies) and 1:20000 (anti- $\beta$ -actin antibody). Visualization was achieved using an enhanced chemiluminescence substrate kit (Pierce).

**Radiation Inactivation.** Protein samples (permeabilized cells or commercially obtained standard enzymes) in 1.5 mL microfuge tubes were flushed with liquid N<sub>2</sub> for 30 min, stored in a freezer at –80 °C, and transported packed in dry ice pellets. Duplicate samples were maintained on dry ice during irradiation ( $\sim 19700$  rad/min), removed at various times, and stored at –80 °C until the enzyme assay, which was performed as described above. Irradiation was performed at the Centers for Disease Control and Prevention using a Gammacell 220 Excel Irradiator Cobalt 60 radiation source (MDS Nordion, Ottawa, ON). Radiation doses delivered by the Gammacell were calculated for each irradiation date using accurate decay charts for the <sup>60</sup>Co-sealed radiation source: dose = (rads per minute)  $\times$  (minutes of irradiation). Enzymatic activities of enzymes from each irradiated sample were determined. Nox5 activity was measured as described above. Activities of standard enzymes maltose dehydrogenase (MDH), alcohol dehydrogenase (ADH), and  $\beta$ -galactosidase were measured as described previously.<sup>22</sup> MDH activity was measured using pig heart cytoplasmic malate dehydrogenase, assayed in a reaction mixture containing 30 mM Hepes, 50 mM KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, and 0.1 mM NADH (pH 7.5), and the sample was added immediately before incubation. Activity was measured as the linear decrease in absorption at 340 nm after the addition of



freshly made 1 mM oxaloacetate. ADH activity was measured using yeast alcohol dehydrogenase, assayed in a reaction mixture containing 75 mM sodium pyrophosphate, 10 mM glycine, 75 mM semicarbazide, 1 mM EDTA, 1 mM NAD<sup>+</sup>, and 0.04% (v/v) ethanol (pH 8.9). Activity was measured by the production of NADH, monitored at 340 nm.  $\beta$ -Galactosidase activity was measured using *Escherichia coli*  $\beta$ -galactosidase. The assay was performed in a reaction mixture containing 100 mM sodium phosphate, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 40 mM 2-mercaptoethanol, and 2.5 mM *o*-nitrophenyl 8-D-galactoside (pH 7.0). Activity was measured at 420 nm as the production of *o*-nitrophenol.

The functional sizes of MDH, ADH, and  $\beta$ -galactosidase were previously reported as 70, 146, and 464 kDa, respectively. As described in detail elsewhere,<sup>23,24</sup> the relationship between the increasing dose of radiation and the remaining enzyme activity is expressed by the equation  $\log(A_D/A_O) = -kD$ , where  $A_D$  is the activity after a given dose of radiation,  $A_O$  is the starting activity,  $D$  is the dose of radiation, and  $k$  is a constant that is directly proportional to the molecular size of the enzyme. The functional sizes of enzyme standards and of Nox5 were estimated using two different methods. (1) The first is the empirically determined equation of Beauregard and Potier:<sup>25</sup>  $\log M_r = 5.89 - \log D_{37} - 0.0028T$ , where  $D_{37}$  is the dose in megarads at which 37% of the activity remains and  $T$  is the temperature in degrees Celsius at which the samples were irradiated ( $-78.5^\circ\text{C}$  on dry ice). (2) Slopes of  $\log(\text{percent activity})$  versus radiation dose were normalized to the slope of the  $\beta$ -galactosidase standard;<sup>26</sup> relative slopes of standards were plotted versus literature values for functional molecular mass, and a best fit line was determined using linear regression using GraphPad Prism (GraphPad Software, San Diego, CA). The molecular mass of Nox5 was estimated by interpolation on the best fit line.

**Statistical Analysis.** Data are presented as ROS generating activity, read as relative luminescence units (for luminol), relative fluorescence units (for Amplex Red), or absorbance at 550 nm (for cytochrome *c* reduction), and expressed as means  $\pm$  the standard deviation (SD). Means were calculated from at least three independent transfection experiments, and each assay was performed in triplicate for each transfection. Statistical significance was determined by a *t* test with GraphPad Prism.

## RESULTS

### Inactive Mutants of Nox5 Inhibit Wild-Type Nox5 Activity.

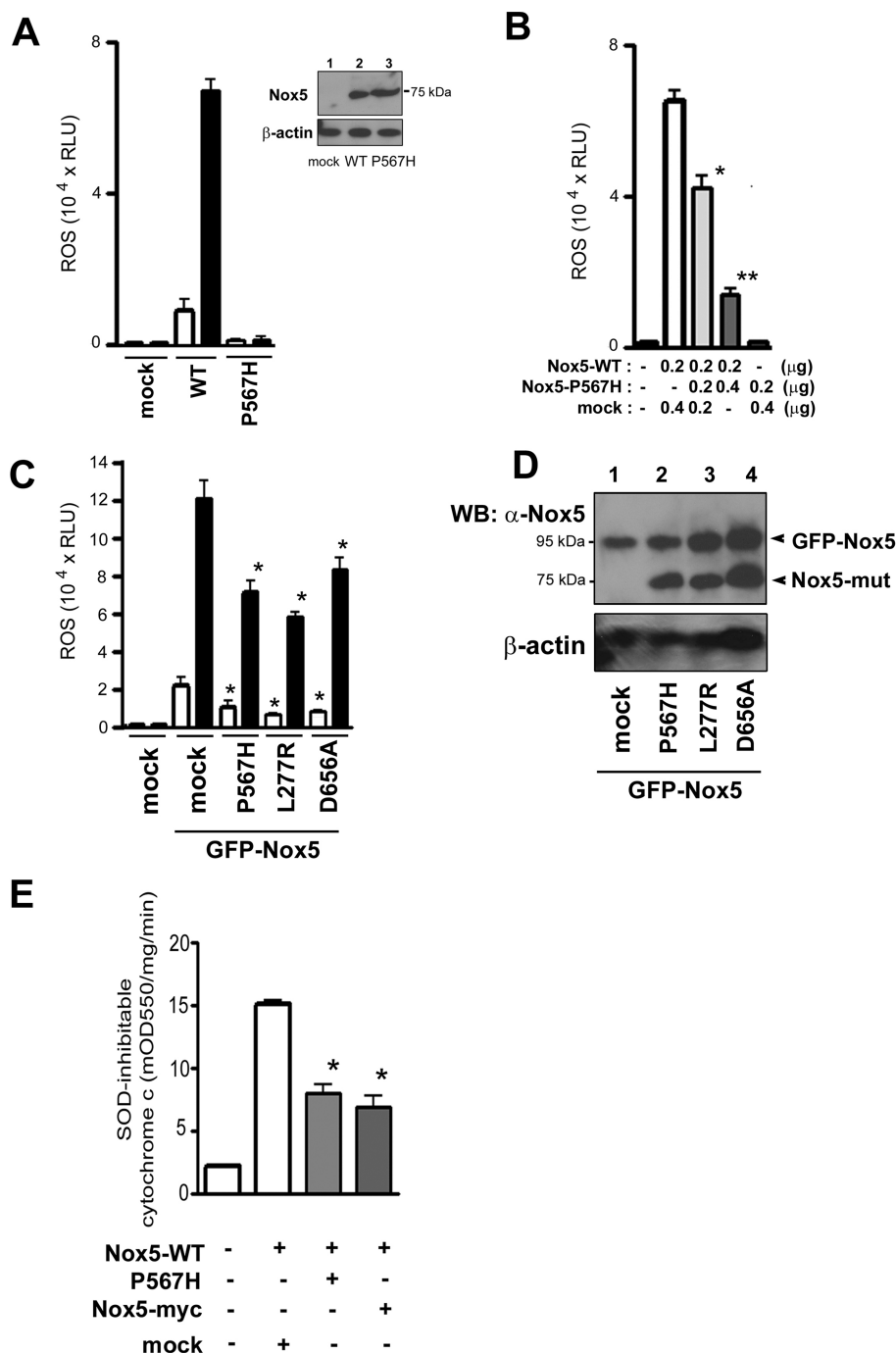
Proline 415 of Nox2, when mutated to a histidine, is expressed in normal amounts but cannot bind NADPH and is therefore inactive.<sup>27–29</sup> The homologous position in Nox5 $\alpha$  is proline 567, which we mutated to generate a catalytically inactive form of Nox5. Human Nox5 $\alpha$  expressed in HEK293 cells generates ROS in response to the calcium ionophore ionomycin (Figure 1A, WT, filled bar). As predicted, Nox5-P567H was expressed at the same levels as wild-type Nox5 but was completely inactive, in both basal and ionomycin-stimulated activity (Figure 1A, P567H). Unexpectedly, coexpression of Nox5-P567H along with wild-type Nox5 resulted in a dose-dependent inhibition of wild-type activity (Figure 1B), thus conforming to the genetic definition of a dominant negative effect. In addition, dominant negative effects were also seen between the same isoenzyme forms using Nox1, Nox2, and Nox4 combined with their homologous NADPH-binding site mutations (T. Kawahara and J. D.

Lambeth, unpublished data). We used the Nox5 isoenzyme to investigate the mechanism of dominant negative inhibition, because Nox5 does not require any known binding or regulatory partner proteins, thereby simplifying interpretation of the data.

An N-terminally GFP-tagged Nox5 construct and native Nox5 previously had been shown to have equivalent activity.<sup>20</sup> We used the larger molecular mass of the GFP-tagged Nox5 to assess protein coexpression of wild-type Nox5 versus mutant Nox5. Like its nontagged version, GFP-tagged wild-type Nox5 exhibited high ionomycin-stimulated activity, which was inhibited by coexpression of Nox5-P567H (Figure 1C); the mutant Nox5 did not decrease the level of expression of GFP-Nox5 (Figure 1D), indicating that the activity effect was not due to suppressed protein expression. To investigate the generality of this effect, we generated additional point-mutated forms of Nox5, selecting residues that were evolutionarily conserved in all EF-hand-containing Noxes from plants to vertebrates.<sup>2</sup> These residues are V276 and L277, both located in the B-loop that joins the second and third transmembrane helices; P438, located in a region between the sixth transmembrane region and the predicted FAD binding site; and D656 and K658, located in the dehydrogenase domain between the second and third predicted NADPH-binding subregions. All these point mutants were expressed at normal levels; mutants L277R and D656A were almost completely inactive (Figure S1 of the Supporting Information). We coexpressed the two inactive mutants with wild-type, GFP-tagged Nox5 and assayed basal and ionomycin-stimulated activity. Like Nox5-P567H, Nox5-L277R and Nox5-D656A inhibited the ROS activity of coexpressed (GFP-tagged) WT Nox5 but did not decrease the level of expression of GFP-Nox5 (Figure 1C,D).

It was unclear from the initial experiments using intact cells whether the inhibition of enzyme activity itself caused the dominant negative responses or whether they might represent indirect effects such as suppressing intracellular calcium ion fluxes or altering the localization of Nox5 at the plasma membrane. To investigate the issue, we measured NADPH-dependent superoxide generation in streptolysin O-permeabilized Nox5-expressing HEK293 cells. The pore size in permeabilized cells is large enough to permit entry of SOD, cytochrome *c*, and calcium ions through the cell membrane into the cytoplasm; consequently, superoxide production is measured in a manner independent of localization, and the intracellular calcium ion concentration can be maintained at  $17\ \mu\text{M}$ .<sup>20</sup> As in intact cells (Figure S2 of the Supporting Information), coexpression of Nox5-P567H suppressed wild-type Nox5 activity in permeabilized cells (Figure 1E). Similarly, a C-terminal expression tag on Nox5 abolishes both basal and calcium-stimulated Nox5 activity.<sup>20</sup> As with the point mutant forms of Nox5, an inactive myc-tagged Nox5 significantly inhibited the activity of GFP-tagged Nox5 in both intact (Figure S2 of the Supporting Information) and permeabilized (Figure 1E) cells. Thus, these results suggest that dominant negative forms of Nox5 affect primarily the activity of the Nox5 enzyme itself and that indirect mechanisms are not responsible for suppression of activity. Among other mechanisms, dominant negative effects can result from formation of dimers or higher-order oligomers that result in an inactive overall conformation of both the active and inactive partner proteins.

**Formation of a Homo-Oligomer of Nox5.** Many examples of membrane-associated proteins whose function requires in-membrane oligomerization exist. These include the formation of



**Figure 1.** Inhibition of wild-type Nox5 by inactive mutants of Nox5. (A) ROS generation was measured in HEK293 cells transfected with vector alone (mock), wild-type Nox5 (WT), or Nox5-P567H (P567H). ROS production was measured by luminol-based luminescence as described in Materials and Methods and expressed as relative luminescence units (RLU) per  $1 \times 10^5$  cells. Cells were treated with DMSO (empty bars) or  $1 \mu\text{M}$  ionomycin (filled bars). Data are means  $\pm$  SD ( $n = 3$ ). The inset shows an immunoblot of the protein samples using an anti-Nox5 antibody or  $\beta$ -actin as a loading control: lane 1, mock; lane 2, Nox5 (WT); lane 3, Nox5 (P567H). Similar results were obtained in two independent experiments. (B) Production of ROS by HEK293 cells cotransfected with the indicated amounts of empty vector (mock), wild-type Nox5 (Nox5-WT), or Nox5-P567H (P567H) was measured by luminol luminescence, expressed as RLU per  $1 \times 10^5$  cells. Cells were activated with  $1 \mu\text{M}$  ionomycin. Data are means  $\pm$  SD ( $n = 4$ ). Compared with Nox5 WT and mock,  $P < 0.05$  (one asterisk) and  $P < 0.005$  (two asterisks). (C) ROS generation (luminol luminescence) by coexpression of GFP-tagged wild-type Nox5 with Nox5 mutation P567H, L277R, or D656A. Equal amounts ( $0.2 \mu\text{g}$  each) of DNA encoding GFP-Nox5 and inactive mutations were used. Cells were treated with DMSO (empty bars) or  $1 \mu\text{M}$  ionomycin (filled bars). Values are means  $\pm$  SD ( $n = 6$ );  $P < 0.05$ , compared with GFP-Nox5 and mock. (D) Protein expression of GFP-tagged wild-type Nox5 and nontagged mutant forms of Nox5 was assessed with immunoblots using an anti-Nox5 antibody. Protein loading was assessed with immunoblots using an antibody to  $\beta$ -actin. Similar results were obtained in two independent experiments. (E) HEK293 cells transfected with  $0.2 \mu\text{g}$  of wild-type Nox5 and  $0.4 \mu\text{g}$  of either Nox5-P567H (P567H), Nox5-myc, or empty vector were permeabilized as described in Materials and Methods and used to measure SOD-inhibitable cytochrome *c* reduction activated by  $17 \mu\text{M}$  free calcium ion. Data are means  $\pm$  SD ( $n = 4$ );  $P < 0.05$ , compared with Nox5 WT and mock.

tetramers by voltage-gated potassium channels,<sup>30</sup> transient receptor potential cation channels,<sup>31–33</sup> and ligand-dependent dimerization of epidermal growth factor receptor.<sup>34</sup> On the basis of the dominant inhibitory effects described above, we hypothesized that Nox5 requires homo-oligomerization for activity. To test this possibility, we coexpressed myc-tagged Nox5 and GFP-tagged Nox5 in HEK293 cells. Immunoprecipitation using an anti-GFP antibody resulted in pull-down of not only GFP-tagged Nox5 but also myc-tagged Nox5 (Figure 2A), demonstrating association of the two proteins. Coexpression of GFP alone with Nox5-myc followed by immunoprecipitation with an anti-GFP antibody failed to pull down Nox5-myc. Similarly, untagged versions of both wild-type Nox5 and the dominant inhibitory Nox5-P567H also co-immunoprecipitated with GFP-tagged Nox5 using an anti-GFP antibody (Figure 2B). The two other untagged dominant inhibitory Nox5 mutants shown in Figure 1C (V277R and D656A) also co-immunoprecipitated with GFP-Nox5 (data not shown). Thus, Nox5 protein forms a homo-oligomer, providing a likely explanation for the inhibitory effects of inactive forms of Nox5.

Because Nox5 is activated by calcium, we tested the hypothesis that calcium might regulate oligomerization, resulting in an active quaternary structure for the enzyme. Co-immunoprecipitation analyses demonstrated that GFP-tagged Nox5 coprecipitated with myc-tagged Nox5 equally well in the presence and absence of 1 mM EDTA (Figure 2C). Thus, Nox5 oligomerization was not affected by calcium ion.

**Absence of the p22phox Subunit in the Nox5 Complex.** Nox1–4 belong to the p22phox-dependent subgroup of Nox enzymes in which the Nox catalytic subunit forms a heterocomplex with p22phox, affecting the stability of the catalytic unit and providing a docking site for regulatory subunits.<sup>8,35,36</sup> Nox5 expression and activity are independent of p22phox,<sup>8</sup> but one study reported that overexpressed p22phox bound to Nox5.<sup>12</sup> To rule out the possibility that p22phox might be a partner in the Nox5 oligomer, a side-by-side experiment was conducted using Nox2 versus Nox5 to test whether each isoform could immunoprecipitate with p22phox. p22phox was readily detected during co-immunoprecipitation with Nox2 (Figure 2D). However, Nox5 did not pull down any detectable p22phox protein. This is consistent with previous observations of the lack of effects of depletion of p22phox on Nox5 activity and shows that Nox5 fails to form a detectable heterocomplex with p22phox. Therefore, oligomerization of Nox5 does not involve participation of p22phox.

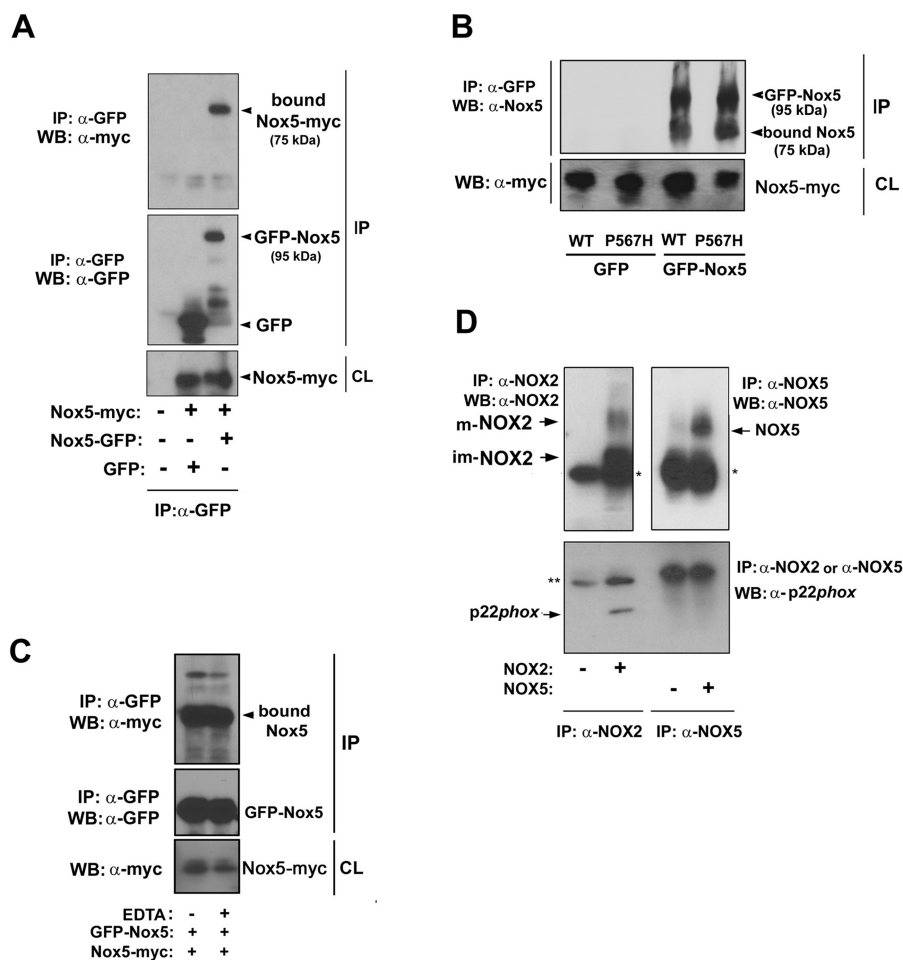
**Role of the Nox5 DH Domain in Oligomer Formation.** Nox5 protein can be divided into four functional domains (Figure 3A): the four EF-hand-containing motifs, the N-terminal polybasic region (PBRN) that regulates subcellular localization of Nox5 through its interaction with phospholipids,<sup>20</sup> the transmembrane, heme-containing domain composed of six  $\alpha$ -helices, and the FAD/NADPH-binding dehydrogenase (DH) domain.<sup>37</sup> To identify the domain that mediates Nox5 oligomerization, we prepared four N-terminally GFP-tagged deletion mutants: (1) Nox5 (S59–F737), from which only the first EF-hand motif was deleted; (2) Nox5 (M173–F737), from which the entire EF-hand domain containing all four EF-hand motifs was deleted; (3) Nox5 (A202–F737), from which the EF-hand domain and PBRN were deleted; and (4) Nox5 (A202–K429), from which the EF-hand domains, the PBRN domains, and the DH domain were all deleted (i.e., only the transmembrane domain remains). All four truncated forms were inactive (both basal and ionomycin-stimulated activity) when expressed in cells

(data not shown). Coexpression of the three deletion mutants containing the DH domain with wild-type Nox5 resulted in inhibition of ionomycin-stimulated Nox5 activity without a change in the expression of full-length wild-type Nox5 protein (Figure 3B). In contrast, removal of the DH domain eliminated the ability of Nox5 (A202–K429) to inhibit wild-type activity. These data suggest that the EF-hand and PBRN regions are not crucial for oligomerization of Nox5 but that the DH domain is a candidate for mediating oligomerization.

The deletion mutants were further tested for their ability to form a complex with full-length Nox5. The GFP-tagged truncations Nox5 (S59–F737), Nox5 (M173–F737), Nox5 (A202–F737), and Nox5 (A202–K429) were coexpressed with myc-tagged full length Nox5 and immunoprecipitated using an anti-GFP antibody. All of the truncation mutants containing the DH domain formed a complex with full-length Nox5 (Figure 3C,D). In contrast, Nox5 (A202–K429), which lacked the DH domain, failed to form a complex with full-length Nox5 (Figure 3D). All of the truncation mutants were expressed approximately equally, and none affected the expression of full-length Nox5 (bottom panel, Figure 3D). Thus, the DH domain must be present to form an oligomer of Nox5, and the ability of truncation mutants to function as dominant inhibitors paralleled their ability to bind to full-length Nox5.

**Essential Role of the DH Domain in the Interaction of Nox5.** To test whether the DH domain is sufficient to form an oligomer, chimeric vectors were designed in which the Nox5 DH domain was linked at the N-terminus to the transmembrane domain of *C. intestinalis* voltage sensor-containing phosphatase (CiVSP), as shown in Figure 4A. In the chimera, the catalytic phosphatase domain of CiVSP was replaced with the DH domain of Nox5, now joined to the transmembrane region of CiVSP, which is comprised of four predicted transmembrane  $\alpha$ -helices. The expressed CiVSP protein exists as a monomer,<sup>38</sup> so any oligomerization must be due to the Nox5 DH domain. Two different CiVSP/Nox5 chimera were generated, one with a C-terminal myc tag and the other with a N-terminal GFP tag. Immunoprecipitation with an anti-myc antibody was then used to test whether the GFP-tagged material was co-immunoprecipitated. As shown in Figure 4B (lane 3), the GFP-tagged chimera containing Nox5-DH coprecipitated with the myc-tagged chimera. The GFP-tagged full-length CiVSP (GFP-CiVSP) bound only slightly to the myc-tagged chimera (Figure 4B, lane 2). The doublet (*b* in Figure 4B) or triplet (*d* and *e*) bands seen in the CiVSP-N/Nox5-DH-C chimeras can plausibly be explained by the introduction or exposure of proteolysis sites in the chimeric protein. Interaction between two full-length Nox5 proteins was shown as a positive control [GFP-Nox5 and Nox5-myc (Figure 4B, lane 4)]. Consistent with previous reports, CiVSP tagged with GFP did not interact with CiVSP tagged with myc (data not shown). Immunoprecipitation with an anti-GFP antibody was performed to show expression of GFP-tagged proteins [the bottom panel of Figure 4B, GFP-CiVSP (lanes 1 and 2), GFP-chimera (lane 3), and GFP-Nox5 (lane 4)]. Therefore, in a membrane-anchored form that uses a heterologous noninteracting transmembrane domain, the Nox5 DH domain self-associates to form an oligomer.

We also tested whether the CiVSP-Nox5 chimera functioned as a dominant negative form by inhibiting the activity of wild-type Nox5. Cells coexpressing wild-type Nox5 and the chimera showed an ~50% decreased level of ROS generation (Figure 4C), whereas those expressing CiVSP and wild-type Nox5 exhibited



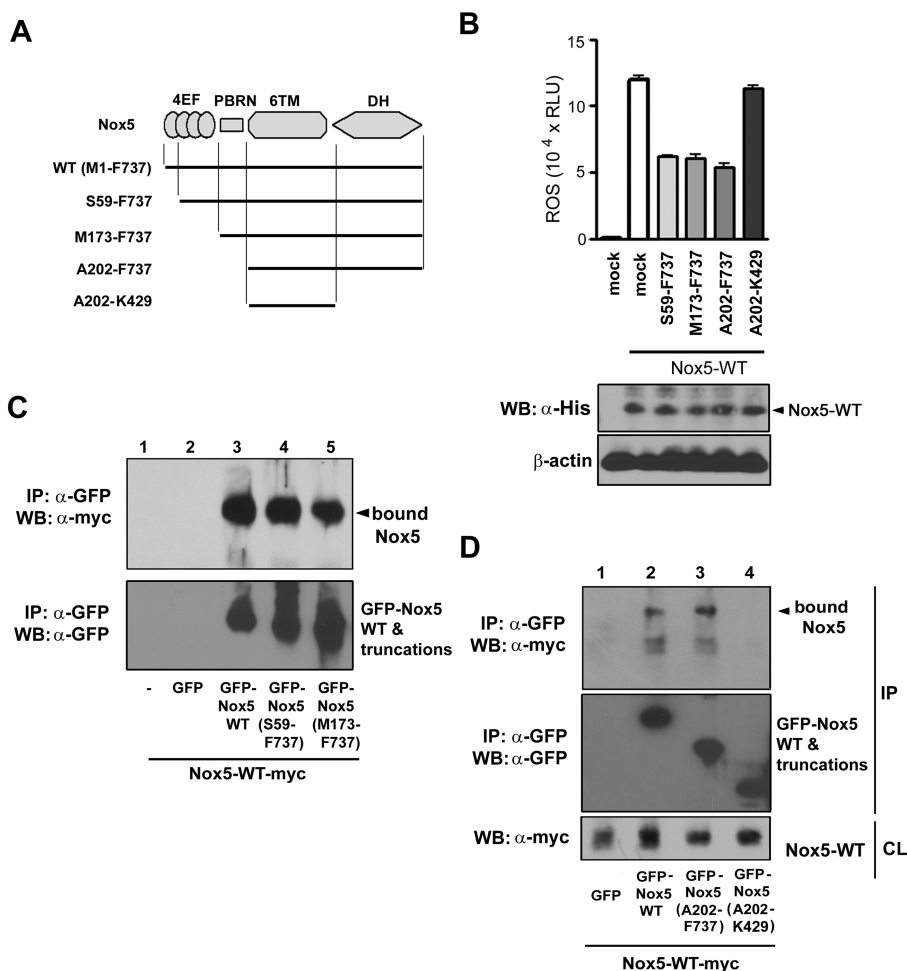
**Figure 2.** Co-immunoprecipitation of Nox5 with itself. (A) Equal amounts (5  $\mu$ g each to a 10 cm dish) of expression vectors encoding myc-tagged Nox5 and GFP-tagged Nox5 were cotransfected in HEK293 cells. The cell lysate was immunoprecipitated with an anti-GFP antibody, and proteins were resolved via SDS–PAGE and immunoblotted with an anti-myc antibody (top) or an anti-GFP antibody (middle). Nox5-myc expression in the cell lysate (CL) was demonstrated with immunoblots using an anti-myc antibody (bottom). (B) myc-tagged wild-type Nox5 or myc-tagged Nox5-P567H was coexpressed along with GFP-tagged Nox5 or GFP alone in HEK293 cells. The cell lysates were immunoprecipitated with an anti-GFP antibody, and proteins were resolved via SDS–PAGE and immunoblotted with an anti-Nox5 antibody (top). Expression of myc-Nox5 in the cell lysate (CL) was demonstrated with immunoblots using an anti-myc antibody (bottom). (C) myc-tagged Nox5 and GFP-tagged Nox5 were coexpressed and immunoprecipitated with an anti-GFP antibody in the presence or absence of 1 mM EDTA (EDTA + and –, respectively). Proteins were resolved via SDS–PAGE and immunoblotted with an anti-myc antibody (top) and an anti-GFP antibody (middle). Nox5-myc expression in the cell lysate (CL) was demonstrated with immunoblots using an anti-myc antibody (bottom). (D) Equal amounts of cDNA encoding Nox2 or Nox5 were transfected in HEK293 cells. Cell lysates were immunoprecipitated with an anti-Nox2 antibody or anti-Nox5 antibody, and proteins were resolved via SDS–PAGE and immunoblotted with an anti-Nox2 antibody (top left panel), an anti-GFP antibody (top right panel), and an anti-p22phox antibody (bottom panel). One asterisk denotes the IgG heavy chain. Two asterisks denote the IgG light chain. Abbreviations: im-Nox2, immature Nox2; m-Nox2, mature Nox2.

only a ~10–15% decrease in activity. This weak inhibition might be due to an effect of the phospholipid phosphatase activity of holo-CiVSP,<sup>39</sup> because phosphatidylinositol 4,5-bisphosphate regulates Nox5 ROS generation by regulating its localization to the plasma membrane.<sup>20</sup> Alternatively, there might be weak binding between GFP-CiVSP and Nox5 DH. Whatever the explanation, the effect is small compared with that of inhibition by the chimera containing the Nox5 DH domain. Expression of full-length Nox5 was not affected by the coexpressed full-length CiVSP or by chimeric protein (Figure 4C, bottom panel). Therefore, the DH domain mediates both oligomerization of Nox5 and its ability to function as a dominant negative inhibitor of wild-type Nox5.

**Estimated Size of the Nox5 Functional Unit in Intact Membranes.** Radiation inactivation has been used to calculate the molecular size of the active enzymatic unit, which may be

a monomer or a multimer, of both soluble and membrane-associated enzymes.<sup>24,26</sup> The theoretical basis for the method has been extensively discussed<sup>23,40,41</sup> and is based on the concept that high-energy radiation will produce dose-dependent random inactivating modifications in enzyme amino acid residues in proportion to the size of the active unit. Thus, if a monomer is active within a larger oligomer, the monomer molecular size will be reported, whereas if the multimer is needed for activity, then the multimer size will be reported. The method has the advantage that enzyme size can be characterized in situ (e.g., membranes or whole cells), without the necessity of perturbing the system, for example by adding detergents. Proteins whose size and oligomerization state are well-known were used as controls: these are MDH (70 kDa, dimer), ADH (146 kDa, dimer), and  $\beta$ -galactosidase (464 kDa, tetramer). These frozen standard enzymes were





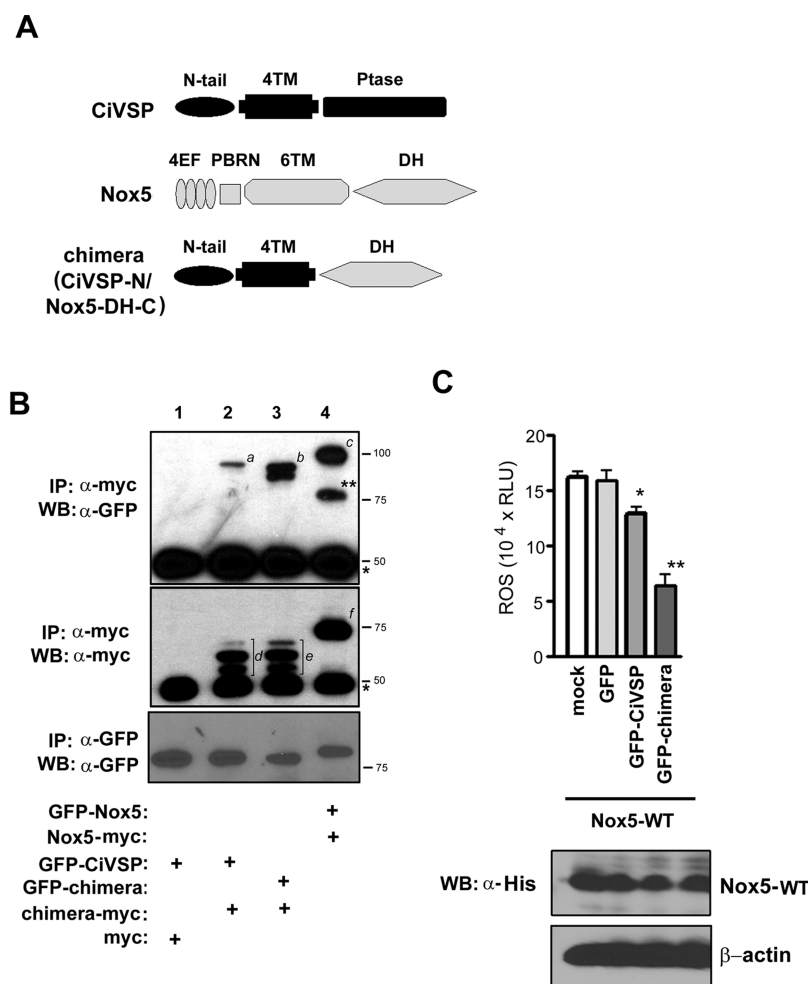
**Figure 3.** Effects of truncated forms of Nox5 on ROS generation by and binding to full-length Nox5. (A) Diagram of truncated forms of Nox5. Abbreviations: 4EF, four-EF-hand-containing calcium-binding domain; PBRN, N-terminal polybasic region; 6TM, six-transmembrane region; DH, dehydrogenase domain. All truncated forms encoded GFP at their N-termini. (B) Expression vectors encoding the indicated truncated Nox5 forms (0.4  $\mu$ g) were cotransfected with N-terminally hexahistidine-tagged full-length Nox5 $\alpha$  (Nox5-WT, 0.25  $\mu$ g) in HEK293 cells. ROS production (luminol luminescence) was measured as described in Materials and Methods. The bottom panels show protein expression of WT Nox5 using an anti-hexahistidine-tagged antibody ( $\alpha$ -His), and protein loading was assessed using an antibody to  $\beta$ -actin. (C and D) Expression vectors encoding the myc-tagged full-length Nox5 and the indicated GFP-tagged truncations of Nox5 were cotransfected into HEK293 cells. Cell lysates were immunoprecipitated with an anti-GFP antibody, and the proteins were resolved via SDS–PAGE and immunoblotted with an anti-myc antibody (top panel in panels C and D) and an anti-GFP antibody (bottom panel in panel C and middle panel in panel D). Nox5-myc expression was evaluated in the cell lysate (CL) with immunoblots using an anti-myc antibody (bottom panel of panel D). The data are representative of three separate experiments.

exposed to radiation for various times over the course of 6 h, and their activities were later determined as described in Materials and Methods. Rates of inactivation are shown in Figure 5, which demonstrates that larger enzymes are inactivated more rapidly than smaller ones. The inset of Figure 5 illustrates this relationship in a standard curve relating functional molecular size to radiation dose. For determination of the size of Nox5, frozen, permeabilized HEK293 cells expressing Nox5 $\alpha$  were exposed to radiation, and calcium-dependent ROS generation was assessed. Figure 5 shows that Nox5 (monomer  $M_r$  = 84 kDa) was inactivated much more rapidly than MDH (70 kDa), and comparison with the standards revealed a molecular size of  $\sim$ 350 kDa. Separate radiation inactivation experiments and different activity assays (cytochrome *c* reduction and Amplex Red fluorescence) for measuring Nox5 activity revealed approximately 30% variability in the size estimates obtained, but all methods yielded size estimates of  $>$ 300 kDa. These data are most consistent with a tetramer ( $n$  = 4.2), assuming that no other

protein participates as a catalytically essential component of the complex.

To ensure that the multimeric functional size did not result from use of an overexpression system, we used a cell system that expresses Nox5 endogenously. Primary cultured proliferating human aortic smooth muscle cells (HASMCs) express both Nox5 $\alpha$  (83.8 kDa monomer size) and Nox5 $\beta$  (82 kDa monomer size) as major forms along with minor amounts of Nox5 $\gamma$  (86 kDa) and Nox5 $\delta$  (85 kDa).<sup>10</sup> It was previously shown that HASMCs produce ROS in response to an increased level of calcium and that the source of the calcium ion-dependent ROS generation was Nox5, based on inhibition by siRNA.<sup>10</sup> We confirmed that HASMCs grown in the presence of PDGF produced ROS that was dependent upon calcium, and that siRNA of Nox5, but not a control siRNA, significantly suppressed expression and activity (Figures S3 and S4 of the Supporting Information). Streptolysin O-permeabilized HASMCs were frozen and irradiated along with standard proteins, and calcium-





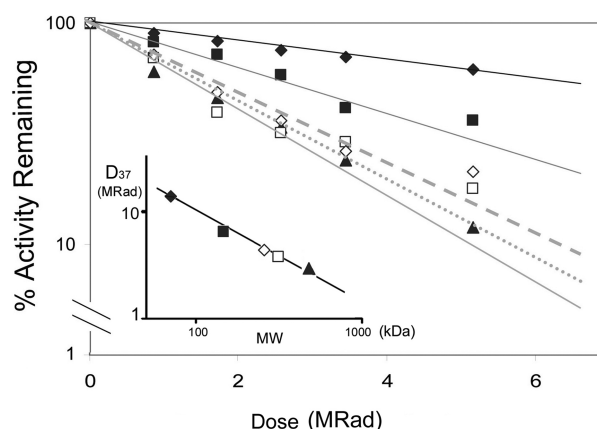
**Figure 4.** Oligomerization is mediated by the Nox5 dehydrogenase domain. (A) Schematic diagram of full-length CiVSP (CiVSP), full-length Nox5 (Nox5), and a chimera comprised of the transmembrane (TM) portion of CiVSP fused to the Nox5 DH domain (chimera, CiVSP-N/Nox5-DH-C). Abbreviations: N-tail, N-terminal cytoplasmic tail region of CiVSP; 4TM, four-helix transmembrane region of CiVSP; Ptase, phosphatase domain of CiVSP. Other abbreviations are as described in the legend of Figure 3A. (B) Equal amounts (5  $\mu$ g) of the indicated vector were cotransfected into HEK293 cells. Lysates were immunoprecipitated using an anti-myc monoclonal antibody, resolved via SDS–PAGE, and immunoblotted with an anti-GFP antibody (top) and an anti-myc antibody (middle). The asterisk denotes the IgG heavy chain. Italic letters (*a–f*) refer to immunostaining protein bands: *a*, GFP-CiVSP; *b*, GFP-chimera (CiVSP-N/Nox5-DH-C); *c*, GFP-Nox5; *d* and *e*, chimera-myc (CiVSP-N/Nox5-DH-C); *f*, Nox5-myc. A protein band appeared around 77 kDa in lane 4 (two asterisks) and is the degradative product of GFP-Nox5 protein. To evaluate the expression of GFP-tagged proteins in the cell lysate, GFP-fused proteins were immunoprecipitated using an anti-GFP monoclonal antibody, resolved via SDS–PAGE, and immunoblotted using an anti-GFP antibody (bottom). Similar results were obtained in four independent experiments. (C) ROS production in HEK293 cells cotransfected with empty vector (mock), full-length, N-terminally hexahistidine-tagged Nox5 (Nox5-WT), and full-length, N-terminally GFP-tagged CiVSP (GFP-CiVSP) or the chimera consisting of the N-terminally GFP-tagged CiVSP TM domain linked to the Nox5 DH domain [GFP-chimera (CiVSP-N/Nox5-DH-C)] was measured using luminol luminescence. Equal expression of full-length hexahistidine-tagged Nox5 (Nox5-WT) in cell lysates (CL) was evaluated using immunoblotting with an anti-hexahistidine-tagged antibody ( $\alpha$ -His, bottom), and protein loading was assessed with immunoblots using an antibody to  $\beta$ -actin. Data are means  $\pm$  SD ( $n = 4$ ); compared with Nox5 WT + GFP,  $P < 0.05$  (one asterisk) and  $P < 0.01$  (two asterisks). Similar results were obtained in two independent experiments.

dependent activity was measured [Figure 5 (diamonds)]. The inactivation rate was similar to that seen in the HEK293 cells, clearly indicating an oligomer as the minimal functional unit, and most consistent with a tetramer. These results also reinforce conclusions based on functional analysis and co-immunoprecipitation experiments that show that active Nox5 functions as an oligomer.

## DISCUSSION

The key finding of this study is that Nox5 forms an oligomer, most likely a tetramer, and that oligomerization is needed for full activity. Furthermore, the oligomerization was mediated by the

dehydrogenase domain. Oligomerization was demonstrated by the following approaches. First, we showed that inactive mutants of Nox5 inhibited calcium-activated ROS generation by wild-type Nox5. While this sort of dominant negative effect does not itself prove oligomerization, this is one of several possible explanations. Second, Nox5 immunoprecipitated with itself, demonstrated by immunoprecipitation of epitope-tagged Nox5 with either wild-type Nox5 or a different epitope-tagged version of Nox5. Third, deletion of the DH domain eliminated oligomer formation and also eliminated the inhibitory effect on Nox5 activity (i.e., the ability of dominant negative forms of Nox5 to inhibit depended on the occurrence of oligomer formation).



**Figure 5.** Radiation inactivation of overexpressed and endogenously expressed Nox5. Frozen samples of permeabilized HEK293 cells transfected with Nox5 ( $\square$ ) or of human vascular smooth muscle cells containing endogenous Nox5 ( $\diamond$ ) were irradiated for the indicated times as described in Materials and Methods. Frozen standard proteins of known functional molecular size were irradiated at the same time: maltose dehydrogenase [70 kDa ( $\blacklozenge$ )], alcohol dehydrogenase [146 kDa ( $\blacksquare$ )], and  $\beta$ -galactosidase [464 kDa ( $\blacktriangle$ )]. Enzymatic activity was assayed in the thawed samples as detailed in Materials and Methods and is expressed as the percent activity remaining after a given dose of irradiation. The inset shows a standard curve of molecular mass (log MW) vs log  $D_{37}$  that is the dose in megarads at which 37% of the activity remains, which allows calculation of a functional molecular mass of Nox5 in HEK cells ( $\square$ ) of 350 kDa and that of Nox5 in vascular smooth muscle cells of 300 kDa, corresponding to calculated oligomer sizes ( $n$ ) of 4.3 and 3.7, respectively. The result was representative of four independent experiments.

Fourth, the DH domain of Nox5 anchored to a heterologous transmembrane domain (from CiVSP) mediates oligomerization, whereas the CiVSP transmembrane domain alone is monomeric. In addition, the CiVSP-linked Nox5 DH domain inhibited wild-type Nox5 activity, providing convincing evidence that the DH domain is a key molecular determinant mediating Nox5 oligomerization. Fifth, radiation inactivation analysis in whole permeabilized cells revealed that the functional Nox5 size of both overexpressed and endogenous Nox5 was most consistent with a tetramer and was much larger than the monomer molecular mass. Thus, Nox5 forms a functionally important oligomer within the membrane, and this oligomer is critically important for calcium-activated ROS generation by Nox5.

The calculation of a tetrameric size for the active form of Nox5 assumes that no additional proteins are present in a catalytically essential complex with Nox5. Indeed, Nox5 was selected for this study because it is known not to require any additional subunits such as regulatory subunits or p22 $phox$ . However, in this context, two additional proteins, c-Abl and calmodulin, are reported to stimulate Nox5 activity in response to  $H_2O_2$  and elevated calcium levels, respectively, and to form complexes with Nox5 under certain activation conditions.<sup>15,42</sup> In the case of c-Abl,  $H_2O_2$  stimulates a calcium flux and tyrosine phosphorylation of c-Abl and results in its translocation to a membrane compartment containing Nox5. In addition, after cells are treated with  $H_2O_2$ , immunoprecipitation of c-Abl results in co-immunoprecipitation of Nox5. However, both the irradiation inactivation and immunoprecipitation experiments described above were conducted without  $H_2O_2$  stimulation of the cells, so significant complex formation with c-Abl would not have occurred under

our conditions. Likewise, ionomycin was used only to assay activity, and calcium-free conditions were used for both co-immunoprecipitation experiments and radiation inactivation experiments; therefore, calcium-induced complex formation with calmodulin is not expected to be an issue under our conditions. Thus, in addition to the *phox*-like regulatory proteins, calmodulin and c-Abl can be ruled out as members of the Nox5 complex demonstrated here.

One possible explanation for dominant inhibitory effects is that the inactive Nox5 binds to signaling proteins such as calmodulin or c-Abl, described above, or protein kinase C, which decreases the concentration needed to activate Nox5. However, in the case of ionomycin activation, a high calcium concentration ( $\sim 1$  mM) fully activates Nox5, and other effectors do not cause further activation.<sup>15,42</sup> In this study, we observed inhibition of Nox5 activity by coexpression of an inactive Nox5 mutant in the ionomycin-treated HEK293 cells, as well as in cells permeabilized in the presence of a maximally activating concentration of free calcium ion. These results demonstrated clearly that the functional effect of the dominant negative forms of Nox5 is not due to binding of the mutant Nox5 to either of these activator proteins.

The molecular details by which oligomerization of Nox5 occurs are not known, but comparison with known oligomeric homologues suggests some possibilities. Our data point to the dehydrogenase domain as the primary interaction site mediating oligomerization. We are not aware of homologous flavoprotein dehydrogenases that have been proven to form oligomers in solution. However, cytochrome  $b_5$  reductase from *Physarum* forms a dimer in the crystal structure.<sup>43</sup> It is not yet clear whether this represents an artifact of crystal packing or a true dimer structure. Nevertheless, the structure provides a reasonable starting point for proposing specific amino acid residues that function in oligomerization. While mutagenesis studies are underway to identify such key residues, we have not yet succeeded in generating an oligomer interface mutant that exists as a monomer. It is therefore not yet possible to test whether the monomer retains some activity. Nevertheless, radiation inactivation experiments imply that the functional unit as it exists in native cells and membranes is an oligomer. In addition, several EF-hand proteins, such as S100 proteins,<sup>44</sup> calpain,<sup>45,46</sup> and polcalins,<sup>47</sup> exist as dimers, and this self-interaction may facilitate regulatory coupling and cooperativity, e.g., upon calcium binding. Often, dimerization in these cases is mediated by noncanonical EF-hand regions that occur as unpaired odd-numbered motifs,<sup>45</sup> and such regions are absent in the Nox5 calcium-binding domain. The recently reported<sup>48</sup> crystal structure of the EF-hand domain of a plant NADPH oxidase, OsRbohB, showed a dimer structure, but it is not clear whether the dimer occurs naturally or is a crystallization artifact. In the case of human Nox5, our study shows that oligomers of Nox5 form in the absence of the EF-hand domain and that the dehydrogenase domain is critical for mediating oligomerization. We cannot rule out the possibility that in an assembled Nox5 oligomer, the calcium-binding domains might also associate in a functionally important manner.

Dimerization and higher-order oligomerization can perform a variety of functions. First, such oligomerization may result in stabilization of the protein, extending its lifetime in the cell. The heterodimer between Nox2 and p22 $phox$ , for example, results in the stabilization of both proteins so that in the absence of either protein, its partner is degraded more rapidly.<sup>49</sup> In addition, oligomerization frequently has regulatory consequences, such

as cooperativity seen for oxygen binding to hemoglobin and substrate binding to many regulated enzymes. We are not aware of any published data that suggest cooperative behavior for regulators or substrates for Nox5, but this possibility cannot be ruled out, as reported experiments were not designed to reveal such kinetic behavior. Finally, inactive forms of Nox may occur naturally and may be involved in adjusting the activity of the active forms of Nox5. Five splice variants of human Nox5 (Nox5 $\alpha$ , - $\beta$ , - $\gamma$ , and - $\delta$  and Nox5S) have been described. Nox5 $\alpha$ , - $\beta$ , - $\gamma$ , and - $\delta$  all include a calcium-binding domain containing all four EF-hand motifs, but with insertions or extensions of different lengths; other regions including the PBRN, transmembrane domain, and DH domain are present in these variants. Nox5S, however, is a variant in which the calcium-binding domain is absent; this form is identical to the truncation mutant M173–F737 from which the calcium-binding domain has been deleted. Nox5S is expressed in human Barrett's adenocarcinoma cell lines where it promotes cell proliferation<sup>50,51</sup> and in human microvascular endothelial cells (HMECs) where it participates in thrombin signaling.<sup>12</sup> Nox5S appears to produce ROS under some circumstances by unknown activation mechanisms but, because it lacks the calcium-binding domain, cannot respond directly to calcium. This study suggests that Nox5S can also function as an endogenous suppressor of calcium-activated forms of Nox5 (i.e., those that retain a calcium-binding domain), because Nox5 (M173–F737) bound to Nox5 $\alpha$  and inhibited its ionomycin-induced ROS generation (Figure 3). HMECs expressed Nox5 $\beta$  (an active long form) as well as significant amount of Nox5S, suggesting that the activity of Nox5 $\beta$  in HMECs might be regulated by the protein level of Nox5S.

In addition, oligomerization could in theory function to concentrate and elevate local concentrations of reactive oxygen within a small area, increasing its effectiveness as a signal molecule, for example, in the regulation of enzymes such as protein tyrosine phosphatases, transcription factors, and ion channels, which has been well documented previously.<sup>1,52</sup> This might be especially effective if the Nox enzyme is colocalized with its target protein in an organelle or membrane subregion. In this context, in vascular smooth muscle, Nox1 has been reported in caveolae, while Nox4 is localized to focal adhesions.<sup>53</sup>

It will be important to address in future studies whether other isoforms of Nox also form oligomers and, if so, the functional consequences. Inactive mutated forms of Nox1, Nox2, and Nox4 also function as dominant negative inhibitors (T. Kawahara, unpublished data), but in these cases, they might act by competing for p22<sup>phox</sup> and/or regulatory subunits. Because of this, it is not straightforward to distinguish this mechanism from one involving homo-oligomerization. Interestingly, cytochrome *b*<sub>558</sub> (the complex formed by Nox2 and p22<sup>phox</sup>), when detergent extracted and purified from human neutrophil membrane, was heterogeneous in size as determined by sedimentation equilibrium analysis, but the smallest detectable species showed a molecular mass of ~350 kDa.<sup>54</sup> If one assumes minimal binding by phospholipids or other bound proteins, then this size corresponds to an oligomer with an *n* of 3–4 (the exact number depending on the contribution of glycosylation to the overall size of gp91<sup>phox</sup>). While it is not clear in this report whether the observed oligomerization occurs naturally or was induced upon detergent extraction of the protein from its native environment, the possibility that oligomerization may be a general property of the Nox family of enzymes and may impact enzymatic function

and ROS signaling by several mechanisms as discussed above should be entertained.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** Additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ■ ABBREVIATIONS

DH, dehydrogenase; CiVSP, *C. intestinalis* voltage sensor-containing phosphatase; ROS, reactive oxygen species; Nox, NADPH oxidase; SOD, superoxide dismutase; TM, transmembrane; EDTA, ethylenediaminetetraacetic acid; HEK, human embryonic kidney; HASMCs, human aortic smooth muscle cells; MDH, maltose dehydrogenase; ADH, alcohol dehydrogenase; HRP, horseradish peroxidase; HMECs, human microvascular endothelial cells.

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