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Involvement of Rac1 in Activation of Multicomponent Nox1- and Nox3-Based NADPH Oxidases

Takehiko Ueyama,[1]* Miklós Geiszt,[2] and Thomas L. Leto[1]*

The Molecular Defenses Section, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892,[1] and Department of Physiology, Semmelweis University, Faculty of Medicine, P.O. Box 259, H-1444 Budapest, Hungary[2]

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Several Nox family NADPH oxidases function as multicomponent enzyme systems. We explored determinants of assembly of the multicomponent oxidases Nox1 and Nox3 and examined the involvement of Rac1 in their regulation. Both enzymes are supported by p47^{phox} and p67^{phox} or homologous regulators called Noxo1 and Noxa1, although Nox3 is less dependent on these cofactors for activity. Plasma membrane targeting of Noxa1 depends on Noxo1, through tail-to-tail interactions between these proteins. Noxa1 can support Nox1 without Noxo1, when targeted to the plasma membrane by fusing membrane-binding sequences from Rac1 (amino acids 183 to 192) to the C terminus of Noxa1. However, membrane targeting of Noxa1 is not sufficient for activation of Nox1. Both the Noxo1-independent and -dependent Nox1 systems involve Rac1, since they are affected by Rac1 mutants or Noxa1 mutants defective in Rac binding or short interfering RNA-mediated Rac1 silencing. Nox1 or Nox3 expression promotes p22^{phox} transport to the plasma membrane, and both oxidases are inhibited by mutations in the p22^{phox} binding sites (SH3 domains) of the Nox organizers (p47^{phox} or Noxo1). Regulation of Nox3 by Rac1 was also evident from the effects of mutant Rac1 or mutant Nox3 activators (p67^{phox} or Noxa1) or Rac1 silencing. In the absence of Nox organizers, the Nox activators (p67^{phox} or Noxa1) colocalize with Rac1 within ruffling membranes, independently of their ability to bind Rac1. Thus, Rac1 regulates both oxidases through the Nox activators, although it does not appear to direct the subcellular localization of these activators.

Reactive oxygen species (ROS) are produced in phagocytic cells by NADPH oxidase, a complex that transports electrons across membranes and generates superoxide anion from molecular oxygen. This enzyme is assembled from a membrane-spanning flavocytochrome b_{558}, composed of Nox2 (also called gp91^{phox}) and p22^{phox} and four cytosolic factors (p47^{phox}, p67^{phox}, p40^{phox}, and Rac) that associate with the flavocytochrome to form an active enzyme (40). Chronic granulomatous disease, characterized by defective microbial killing by phagocytic cells, is caused by defects or deficiencies in any one of four oxidase components: Nox2, p22^{phox}, p47^{phox}, or p67^{phox} (40). An essential role for Rac1 or Rac2 in NADPH oxidase activation was also identified in cell-free reconstitution studies (1, 32). This role was later confirmed in an oxidase-deficient patient who expressed mutant Rac2 (5) and in mice rendered genetically deficient in Rac2 (48). Recently, several novel homologs of the catalytic, electron carrier component of NADPH oxidase (gp91^{phox} or Nox2) have been described in a variety of nonphagocytic cells (22, 36). This new NADPH oxidase or Nox family encompasses seven enzymes identified in humans: Nox1, Nox2 (gp91^{phox}), Nox3, Nox4, Nox5, Duox1, and Duox2. Nox1 and Nox3 are the closest homologs of Nox2 in terms of structure and function. Nox1 is detected in abundance in the colon and at lower levels in uterus, prostate, and vascular smooth muscle and endothelial cells (2, 50). Although originally suggested to function as a promitogenic oxidase, Nox1 more likely functions as a host defense oxidase (19, 29), like Nox2, and may have physiological and pathological roles in vascular tissue (2, 38). Nox3 mRNA was detected in several fetal tissues by reverse transcription-PCR, including kidney, liver, lung, and spleen (12, 31). Recently, a unique role for this oxidase was revealed within the inner ear (vestibular system) by positional cloning studies that mapped genetic lesions causing the head tilt (*het*) phenotype in mice (45). Mice with Nox3 mutations exhibit impaired otoconial morphogenesis and defects in perception of gravity and balance. Subsequently, Nox3 expression was detected in the cochlear system, and it was suggested that Nox3-derived ROS could contribute to hearing loss and balance problems (8).

Both the Nox1 and Nox3 oxidases appear to function as multicomponent enzymes similar to the phox (Nox2-based) system (7, 8, 14, 15, 21, 22, 29, 54, 55). Nox1 expressed alone produces little superoxide, although its activity is dramatically enhanced in the presence of two cofactors (Noxo1 and Noxa1) that are detected in colon epithelium (7, 21, 54). Noxo1 was proposed to act as a "Nox organizer" based on structural and functional similarities with p47^{phox}, which is a multimodular adaptor protein that bridges interactions between the flavocytochrome b_{558} and p67^{phox} by binding to both p22^{phox} and p67^{phox}. Noxa1 was designated as a "Nox activator" based on its homology to p67^{phox}, which binds to Rac1 or Rac2 and promotes electron flow through the flavocytochrome in a GTP-dependent manner (10). The phagocytic oxidase can be reconstituted in vitro in the absence of p47^{phox}, when p67^{phox} and

* Corresponding author. Mailing address for Thomas L. Leto: NIH, NIAID, Twinbrook II, Room 203, 12441 Parklawn Dr., Bethesda, MD 20892. Phone: (301) 402-5120. Fax: (301) 480-1731. E-mail: tleto@nih.gov. Mailing address for Takehiko Ueyama: Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan. Phone: 81-78-803-5962. Fax: 81-78-803-5971. E-mail: tueyama@kobe-u.ac.jp.

Rac1 are provided in excess (18, 34) or when $p67^{phox}$ is adapted with the membrane-binding sequences of Rac1, although GTP-bound Rac is still required for activation (3, 23, 24, 42). Noxa1 was shown to bind Rac1 in a GTP-dependent manner (54), although its role as a Nox1 regulator involving Rac1 has not been demonstrated directly. Both the human and murine Nox1 systems (comprising Nox1, Noxo1, and Noxa1) exhibit high constitutive activity, while activity of the human system is significantly enhanced by cellular activation in several cell types (7, 21, 28, 54). Recently, several groups examined involvement of the Nox1 or Nox2 (phox) regulators in Nox3 activity and showed that this enzyme is less subject to the tight cofactor-dependent controlling mechanisms observed with Nox1 or Nox2 (8, 15, 55). Furthermore, there has been no direct evidence for Rac-dependent regulation of Nox3 activity (55).

In this study we examine the multicomponent nature (subcellular targeting, assembly, and activation) of Nox1- and Nox3-based oxidases in transfected cell models by systematically expressing wild-type and mutated forms of various oxidase components. We present evidence indicating that Nox1 and Nox3 are modulated by Rac1 and that this involves the Rac-binding Nox activators, Noxa1 and $p67^{phox}$. We also show that Noxo1 acts as an adaptor protein targeting Noxa1 to the plasma membrane and that $p22^{phox}$ is targeted to the plasma membrane when either Nox1 or Nox3 is coexpressed. Finally, using a plasma membrane-targeted form of Noxa1, we reconstitute Noxo1-independent Nox1 activity, which is critically dependent on Rac1.

MATERIALS AND METHODS

Materials. Diphenyleneiodonium chloride (DPI) and superoxide dismutase (SOD) were purchased from Sigma-Aldrich and EMD Biosciences, respectively. Mouse monoclonal antibodies (Abs) against human Rac1 and β-tubulin were from Upstate Biosciences and Sigma-Aldrich, respectively. Rabbit polyclonal anti-V5 peptide horseradish peroxidase Ab and rabbit polyclonal anti-green fluorescent protein (anti-GFP) Ab were from Invitrogen and Clontech, respectively. Rabbit polyclonal Ab was raised against recombinant glutathione *S*-transferase (GST) fused to full-length mouse Noxa1, produced in *Escherichia coli* using pGEX-4T1 (Amersham Biosciences). Goat polyclonal Abs against human $p47^{phox}$ and $p67^{phox}$ were described previously (39). Mouse monoclonal Ab against $p22^{phox}$ (no. 449) was a kind gift from Dirk Roos (57). Rac1 Validated Stealth RNAi (RNA interference) DuoPak and Stealth RNAi Negative Control Medium GC Duplex were obtained from Invitrogen, and Rac1 Silencer Predesigned siRNA (short interfering RNA) and Silencer Negative Control no. 1 siRNA were from Ambion.

Cell culture. All cell culture reagents were obtained from Invitrogen, unless indicated otherwise. Human HEK293 cells (ATCC) were maintained in Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories), 1 mM sodium pyruvate, 100 μM nonessential amino acids, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2. CHO-K1 cells (ATCC) were maintained in Ham's F-12 medium containing 10% heat-inactivated FBS and antibiotics at 37°C in 5% CO_2. HT-29 cells (ATCC) were maintained in McCoy's 5A medium (modified) containing 10% heat-inactivated FBS and antibiotics and grown in 5% CO_2 at 37°C.

Construction of plasmids. The pcDNA3.1 plasmids (Invitrogen) containing the complete coding sequence of human Nox1, Nox2, $p51^{nox}$ (Noxa1), $p47^{phox}$, $p67^{phox}$, and $p22^{phox}$ were described previously (20, 21). The full coding sequence for human Nox3 was amplified by PCR using fetal kidney first-strand cDNA (Stratagene) using specific primers designed from previously reported sequence (GenBank accession number NM_015718) and cloned into pcDNA3.1. Human $p41^{nox}$ (Noxo1β) in pcDNA3.1 was made by adding codon Lys^{50} to Noxo1α (21) using the QuikChange II XL site-directed mutagenesis kit (Stratagene). Full-length Noxo1β and a C-terminally truncated mutant of Noxo1β (amino acids [aa] 1 to 319) lacking the proline-rich region (PRR; aa 320 to 329) that interacts with the C-terminal SH3 domain of Noxa1 were amplified by PCR, cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen), and designated Noxo1-V5 and Noxo1 (ΔPRR)-V5, respectively. Full-length Noxo1β was amplified by PCR and cloned into the EcoRI and BamHI sites of pEGFP-N1 (Clontech) and designated Noxo1-GFP. We confirmed that Noxo1-GFP supports Nox1 activity in the presence of Noxa1 at levels comparable to those of unfused, native Noxo1 (data not shown) (14). The cDNAs encoding human wild-type Rac1, Rac1(Q61L), Rac1(T17N), and Rac1(G30S) were described previously (35); these cDNAs were amplified by PCR and cloned into pCMV-Myc (Clontech) to express N-terminal, c-Myc epitope-tagged versions of these proteins. Mouse Rac1 in pEGFP-C1 (Clontech) was described previously (56). Human Noxa1 and $p67^{phox}$ coding sequences were adapted with the C-terminal, polybasic region of Rac1 (KKRKRK; aa 183 to 188) and isoprenylation "CAAX" motif of Rac1 (CLLL; aa 189 to 192) by PCR amplification (see Fig. 2B and Fig. 9D), using specific reverse primers that provided these sequences, and then cloned into pcDNA3.1; the fused constructs were designated Noxa1(pp) and $p67^{phox}$(pp), respectively. All other indicated amino acid mutations, Noxa1(R103E), Noxa1(pp, R103E), Noxa1(W436R), Noxa1(R103E, W436R), Noxo1(W197R), $p67^{phox}$(R102E), $p67^{phox}$(pp, R102E), and $p47^{phox}$(W193R) in pcDNA3.1 and Noxo1(W197R) in pcDNA3.1D/V5-His-TOPO, were produced using the QuickChange II XL site-directed mutagenesis kit. All modified expression vectors were sequenced to confirm their identities.

Cell fractionation and immunoblotting. Cells were sonicated in disruption buffer (44) in the present of protease inhibitor cocktail (Sigma-Aldrich), and the sonicate was centrifuged for 10 min at 10,000 × *g*. The supernatant was further centrifuged for 1 h at 100,000 × *g*. The pellet was defined as the membrane fraction, and the supernatant was defined as the cytosolic fraction. Western blotting was performed on proteins transferred to nitrocellulose membranes, preblocked in 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.03% Triton X-100 (TBST), and probed with primary antibody prepared in TBST at the dilutions indicated: Noxa1, 1:1,000; Rac1, 1:2,000; $p47^{phox}$, 1:2,000; $p67^{phox}$, 1:2,000; β-tubulin, 1:2,000; V5-horseradish peroxidase, 1:5,000; GFP, 1:2,000. Bound antibodies were detected with secondary antibody-peroxidase conjugates using the ECL detection system (Amersham Biosciences). In the case of fractionation studies using synthetic siRNA, suppressed levels of Rac1 protein were confirmed using aliquots of total lysates in each experiment.

Confocal fluorescence imaging studies. Cells (HEK293 or CHO-K1) were seeded on 35-mm glass-bottom dishes (MatTek Chambers) and transfected using FuGENE 6 (Roche Applied Science). At 40 to 48 h after the transfection, cells were fixed using 10% formalin in neutral buffered solution (Sigma-Aldrich). After permeabilization with TBS containing 0.3% Triton X-100 for 10 min, the fixed cells were stained for 2 h at room temperature (RT) using each primary antibody (Noxa1, $p67^{phox}$, $p22^{phox}$, or Rac1) at 1:200 dilutions (TBST with 5% bovine serum albumin). Secondary antibody-fluor conjugates (anti-rabbit antibody–Alexa 488 or anti-mouse or anti-goat antibody–Alexa 594; 1:2,000 dilutions) were applied for 0.5 h at RT. Confocal imaging was performed using a TCS-SP2 ABOS confocal laser scanning fluorescence microscope (63× oil) (Leica Microsystems GmbH).

Rac1 activation assay. Rac1 activation assays were performed using the Rac1 activation assay kit (Upstate Biosciences) according to the manufacturer's suggested protocol. Briefly, cells (HEK293 or CHO-K1) grown in 10-cm dishes were harvested using 500 μl of lysis/wash buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin). Cell lysates were centrifuged for 5 min at 3,000 × *g*, and the resulting supernatants were mixed with 10 μg of GST-PAK1-CRIB conjugated to glutathione-agarose beads and rocked at 4°C for 1 h. For positive and negative control experiments, 100 μM GTPγS or 1 mM GDP was added in 500 μl of the resulting supernatant described above. After the mixture was rocked for 15 min at RT, the supernatant was mixed with GST-PAK1-CRIB-conjugated glutathione-agarose beads and rocked at 4°C for 30 min. After three washes, the beads were resuspended in Laemmli sample buffer, and the proteins bound to PAK1-CRIB were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with Rac1 antibody (Upstate Biosciences).

Cell transfections and ROS production assays. Cells were seeded in six-well dishes at 250,000 cells/well (HEK293 cells), 75,000 cells/well (CHO-K1 cells), or 700,000 cells/well (HT-29 cells) for 48 h prior to transfection. Cell transfections were performed in serum-free medium using 6 μl of FuGENE 6 prepared in complexes with plasmid DNAs (HEK293 and CHO-K1 cells, total, 2 μg/well; 0.5 μg of Nox1 or Nox3, plus 0.5 μg each of other expression vectors or pcDNA3.1 [control] plasmid; HT-29 cells, 0.5 μg each of expression vectors or pcDNA3.1 [control] plasmid without Nox1), using the manufacturer's suggested protocol; in studies expressing mutant products, equal moles of plasmid were used in place of wild type. The cells were fed 5 h posttransfection with complete medium and were assayed 48 h after transfection. Trypsinized cells were assayed for ROS release (with or without activation with 2 μg/ml phorbol myristate acetate

[PMA]) by superoxide dismutase-sensitive chemiluminescence methods using the Diogenes reagent (National Diagnostics), as described previously (21). The reagent has 10,000 times greater sensitivity for superoxide than does hydrogen peroxide (J. Kitzler [National Diagnostics], personal communication).

RNAi-mediated silencing of Rac1. Rac1-specific short hairpin RNAs (shRNAs) were expressed in pSUPER-gfp, a Neo-resistant vector from Oligoengine (Seattle, WA). Four sequences of 19 or 21 nucleotides in length specific for human Rac1 cDNA (GenBank accession number NM_006908) were selected for synthesis of shRNA. Their sequences (5′-ACCGGTGAATCTGGGCTTA-3′, 5′-ACACTC CCATCATCCTAGT-3′, 5′-CCTTTGTACGCTTTGCTCA-3′, and 5′-GCTAAT AAGTGCTTTCCTTAG-3′), corresponding to nucleotides relative to the ATG translational initiation site (147 to 165, 320 to 338, 618 to 636, and 1664 to 1684, respectively) were cloned into BglII and HindIII sites of the vector; these vectors were named pSUPER-Rac1-147, pSUPER-Rac1-320, pSUPER-Rac1-618, and pSUPER-Rac1-1664, respectively. The constructs were sequenced to confirm their identities. In the case of RNAi studies using synthetic siRNAs, the following Rac1-specific or negative control RNAs were used: Rac1 Validated Stealth siRNA-2 (5′-AGGGUCUAGCCAUGGCUAAGGAGAU-3′), Stealth RNAi Negative Control Medium GC, Rac1 Silencer Pre-designed siRNA (siRNA identification: 214526, GCCACUACAACAGAAUUUU), and Silencer Negative Control no. 1 siRNA.

For ROS production assays, HEK293 cell transfections were performed as described above, using 0.1 μg of Nox1 or Nox3, plus 50 ng each of other regulator plasmids, and 2.0 μg of pSUPER-Rac1 or pSUPER-gfp (control) plasmid. In the case of Noxa1(pp), 0.2 μg of Nox1 plus 0.1 μg of Noxa1(pp) and 1.7 μg of pSUPER-Rac1 or pSUPER-gfp (control) plasmid were used. For synthetic siRNA experiments, transfections of plasmid plus siRNAs were performed using 5 μl of Lipofectamine 2000 and Opti-MEM I (Invitrogen), as described above. Suppressed Rac1 protein production was confirmed by immunoblotting, as described above.

Statistical analysis. Data are presented as the percentage of the maximally reconstituted oxidase activities observed in the absence of cell stimulation and were expressed as means ± standard deviations (SD). For Nox1 experiments, full activity (100%) was defined with Nox1 plus Noxo1 plus Noxa1 coexpression; for Nox3, 100% was defined with Nox3 plus Noxo1; for Nox2, 100% was defined with Nox2 plus $p47^{phox}$ plus $p67^{phox}$ plus PMA. Mean activities were calculated from at least three independent transfection experiments, where each assay was performed in duplicate.

RESULTS

Subcellular localization of the Nox activator, Noxa1, is Noxo1 dependent and Rac1 independent. Earlier studies have shown that Nox1 activity requires coexpression of two supportive cofactors, Noxo1 and Noxa1, which are homologous to $p47^{phox}$ and $p67^{phox}$, respectively (7, 14, 21, 54). The activity of the reconstituted human Nox1 system is significantly enhanced by cell stimulation in several transfected models, including NIH 3T3, HEK293, CHO-K1, Cos-7, and T84 cells (21, 29, 54). Recently, Noxo1 was shown to accumulate on the plasma membrane through PX domain-lipid interactions, even without cell stimulation (14). To explore assembly of the Nox1 system further, we examined the subcellular localization of Noxa1 in relation to Noxo1 in transfected HEK293 cells (Fig. 1A to D). Noxa1 was detected with an antibody raised against recombinant murine Noxa1, which detects Noxa1 as a doublet band around 50 kDa by immunoblotting (Fig. 1E), while Noxo1 subcellular localization was followed with a Noxo1-GFP fusion protein. Noxo1-GFP fluorescence occurs predominantly along the plasma membrane (Fig. 1A), as described earlier (14). The association of Noxa1 with the plasma membrane is dependent on Noxo1 (Fig. 1B), since this localization pattern is disrupted by a Noxa1 SH3 domain mutation (W436R) previously shown to inhibit its interactions with the C-terminal PRR of Noxo1 (54). Furthermore, Noxa1 accumulation along the plasma membrane is not observed in the absence of Noxo1 (Fig. 1C). In this case, Noxa1 is found primarily in the cytosol, although a small portion of Noxa1 is also localized to ruffling membranes (Fig. 1C), especially on the upper surfaces of cells (data not shown). The association of Noxa1 with ruffling membranes is not disrupted with mutant Noxa1(R103E) (Fig. 1D), which no longer binds to Rac1 (54). These results were confirmed in cell fractionation experiments, in which the Noxa1 plasma membrane-targeting protein, Noxo1-GFP, is detected entirely within the membrane (particulate) fraction, not in the cytosolic (soluble) fraction (Fig. 1E). In contrast, the control unfused GFP protein is detected solely in the cytosolic fraction (Fig. 1F, right). Although a portion of Noxa1 is detected in the membrane fraction, the amount in this fraction is increased significantly by cotransfection of Noxo1-GFP but not mutant Noxo1(W436R)-GFP (Fig. 1E, left). The distribution of Noxa1 between membrane and cytosolic fractions is unaffected by the R103E mutation; by coexpression of the constitutively active form of Rac1, Rac1(Q61L); or by suppressing Rac1 production using two specific Rac1-targeted siRNAs (Fig. 1F and G). These observations demonstrate that Noxo1 can serve as an adaptor protein mediating interactions between Noxa1 and the plasma membrane, similar to the role of $p47^{phox}$ in the Nox2 system. In addition, these experiments suggest that Rac1 does not affect the subcellular distribution of Noxa1.

Rac1 modulates Nox1 through its binding partner Noxa1. The importance of Noxo1 as a mediator of Noxa1-membrane interactions was then explored in Nox1-reconstituted cells. In the HEK293 cell model, the Diogenes-based luminescence assay of Nox1 activity supported by Noxo1 and Noxa1 coexpression is significantly stimulated by PMA, inhibited by DPI, and completely abolished by extracellular addition of SOD (Fig. 2A). However, Nox1 does not produce significant ROS when expressed alone or in pairwise combinations with Noxo1, Noxa1, or Rac1(Q61L), confirming the previously established model involving both Nox regulators (Fig. 2A). Based on several reports indicating that $p47^{phox}$ is dispensable in cell-free reconstitution of the Nox2 system (18, 34), particularly if $p67^{phox}$ is adapted with membrane-binding sequences of Rac1 (3, 23, 24, 42), we hypothesized that Noxa1 could support Nox1 activity in the absence of its membrane adaptor partner (Noxo1) if Noxa1 is directly targeted to the plasma membrane. Based on reports showing that the polybasic amino acids of Rac1 (KKRKRK; aa 183 to 188) together with the "CAAX" isoprenylation motif (CLLL; aa 189 to 192) can target proteins to the plasma membrane (16, 56), we constructed a plasma membrane-targeted form of Noxa1 involving a C-terminal fusion with these human Rac1 sequences (Fig. 2B). As shown in Fig. 2C, both immunofluorescence and subcellular fractionation experiments confirmed that this modified form of Noxa1 [Noxa1(pp)] is efficiently targeted to the plasma membrane. Noxa1(pp) supports low but detectable levels of ROS release in Nox1-expressing cells in the absence of Noxo1, although cotransfection of Noxa1(pp) along with Rac1(Q61L) dramatically enhances ROS production by Nox1 (Fig. 2A).

To characterize further the activation of Nox1 supported by Noxa1 in the absence of Noxo1, we examined the effects of Noxa1(R103E) and Rac1 mutants. As shown in Fig. 2A, Noxa1 alone does not support detectable Nox1 activity; however, cotransfection of Noxa1 and Rac1(Q61L) does (Fig. 2D). This activity was completely abolished by the Noxa1(R103E) mutation that disrupts Rac1 binding. Noxo1 and Noxa1(pp) together support activity comparable to levels observed with the wild-type

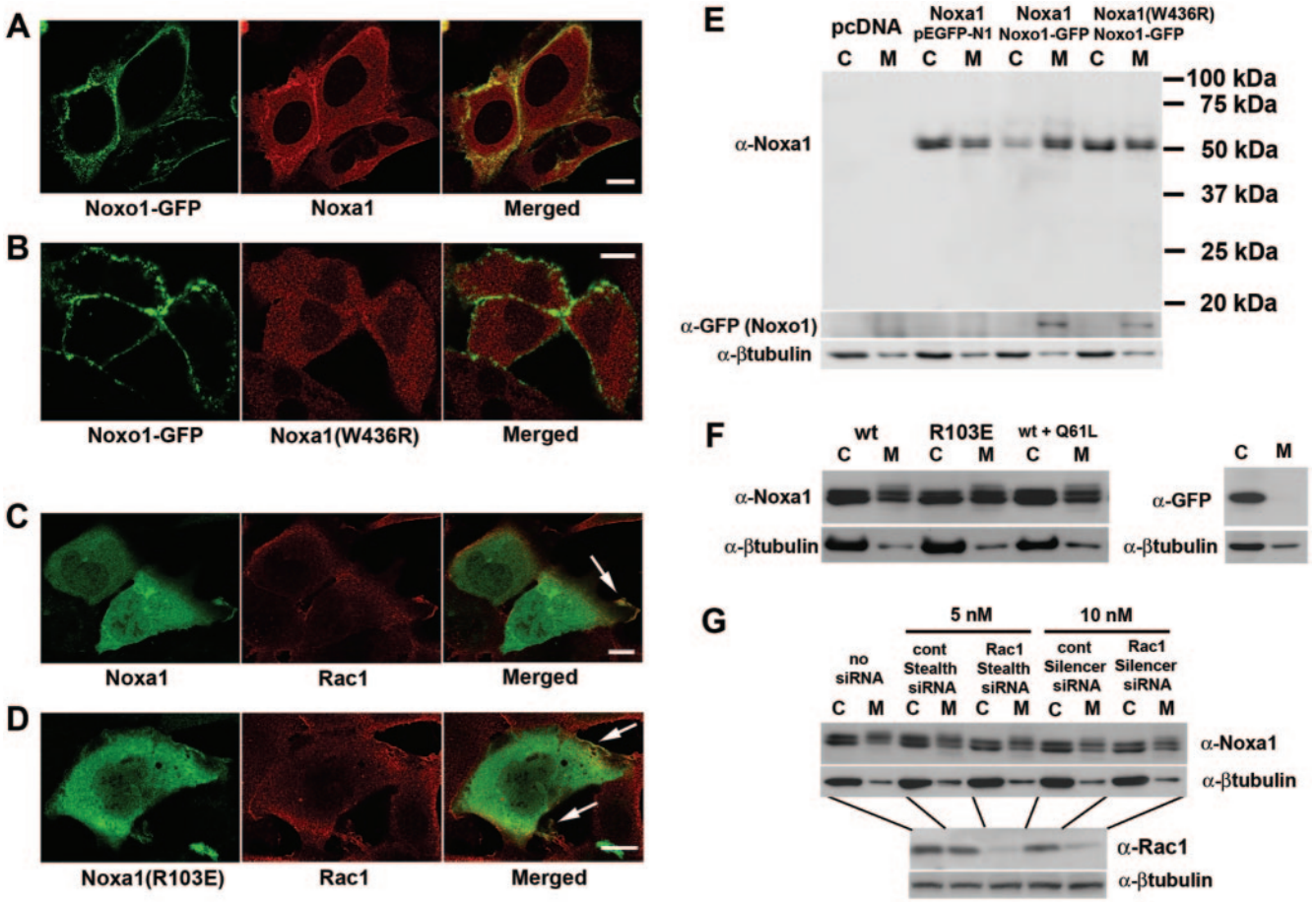

FIG. 1. Noxo1-dependent and Rac1-independent subcellular localization of Noxa1 in HEK293 cells. A. Noxo1-GFP is detected predominantly along the plasma membrane. Coexpressed Noxa1 colocalizes with Noxo1 along the plasma membrane. B. The association of Noxa1 with the plasma membrane is disrupted by an SH3 domain mutation (W436R) that was shown to disrupt its interaction with the C-terminal, proline-rich region of Noxo1. C. Noxa1 expressed alone is localized primarily in the cytosol, although some Noxa1 accumulates at ruffling membranes (arrow). D. Localization of Noxa1 at ruffling membranes is not affected by a mutation [Noxa1(R103E)] that disrupts its interaction with Rac1. E. The Noxa1 antibody detects transfected Noxa1 as a doublet band around 50 kDa. Wild-type Noxa1 is detected in both cytosol and membrane fractions, consistent with fluorescence imaging. Membrane-associated Noxa1 increases by coexpression of Noxo1-GFP but not Noxo1(W436R)-GFP. Plasma membrane localizing protein, Noxo1-GFP, is detected predominantly in the membrane fraction. F. The fractionation of Noxa1 is not affected by a Noxa1 mutation at R103E or coexpression of Rac1(Q61L). Right, cytosolic (unfused) GFP is detected predominantly in the cytosol fraction. G. Fractionation of Noxa1 is not affected by two Rac1-suppressing siRNAs (Stealth and Silencer). Immunoblotting of total lysates confirms effective suppression of endogenous Rac1 by these siRNAs. no siRNA, transfection without siRNA. Bar, 10 μm. Similar protein loading is confirmed by β-tubulin blotting. Similar results were obtained in four (A, B, C, D, and F) or three (E and G) independent experiments.

proteins, although these proteins show a lower PMA-enhanced component (Fig. 2D). Nox1 activity, supported by Noxa1(pp) alone, is also completely abolished with the Noxa1(pp, R103E) mutant. Nox1 activity reconstituted by Noxa1(pp) and Rac1 (Q61L) is significantly reduced with the Noxa1(pp, R103E) mutant. Finally, no activity is detected by substitution with Noxa1(pp, R103E) combined with Rac1(G30S) (Fig. 2D). Thus, our observations demonstrate that Rac1 can modulate Nox1 through interactions with its binding partner, Noxa1. These experiments reveal close functional parallels in the assembly of Nox1- and Nox2-based oxidases, since Rac1 can modulate both systems through activator proteins (Noxa1 or $p67^{phox}$), which in turn require critical adaptor or organizer proteins (Noxo1 or $p47^{phox}$) to link them to the plasma membrane oxidase complex. Our findings on the contribution of Rac1 to Nox1 activation supported by the membrane-targeted form of Noxa1 [Noxa1(pp)] are analogous to observations on Nox2 reconstitution by similar, membrane-binding forms of $p67^{phox}$ (3, 23); targeting of $p67^{phox}$ fusion proteins containing the C-terminal, membrane-binding sequence of Rac1 was not sufficient to support Nox2 in vitro, and addition of the Rac1-GTP moiety is required for full activation.

Rac1 regulates the fully reconstituted Nox1 system. To explore further the involvement of Rac1 in the fully reconstituted system comprising Nox1, Noxo1, and Noxa1, we examined two transfected models, HEK293 and CHO-K1 cells. In the HEK293 cell model, neither the defective effector site mutant, Rac1(G30S), nor the constitutively active mutant, Rac1(Q61L), significantly affects Nox1 activity when reconstituted with both Noxo1 and Noxa1 (Fig. 3A). However, in the reconstituted CHO-K1 cell model, Rac1(G30S) shows dominant-negative effects on both basal and PMA-stimulated Nox1 activities (Fig. 3B). Furthermore, Rac1(Q61L) has an enhancing effect on

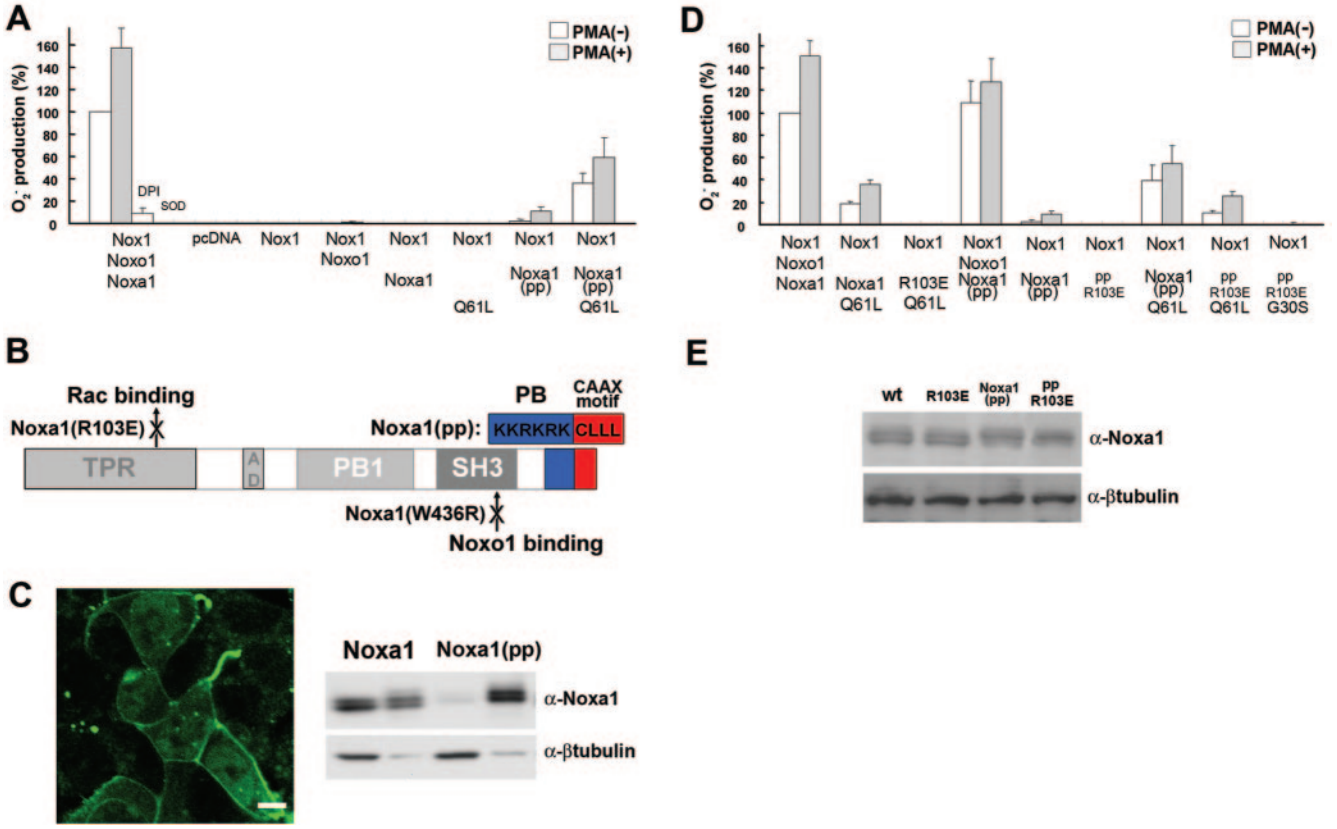

FIG. 2. Nox1 activity supported by membrane-targeted Noxa1 is Rac dependent but Noxo1 independent. A. Optimal reconstitution of Nox1 activity in HEK293 cells requires cotransfection with partners Noxo1 and Noxa1. Cotransfection of Nox1 with Noxo1, Noxa1, or constitutively active Rac1(Q61L) does not support significant activity. Membrane-targeted Noxa1(pp) can partially compensate for the absence of Noxo1; coexpression of Rac1(Q61L) dramatically enhances Nox1 activity supported by Noxa1(pp). Data are from at least three independent transfection experiments. B. Schematic representation of Noxa1(pp) structural features. C. Noxa1(pp) accumulates along the plasma membrane. Bar, 10 μm. Right, cell fractionation confirms Noxa1(pp) localization in the membranes; similar results were obtained in two independent experiments. D. Nox1 activity supported by Noxa1 or Noxa1(pp) is enhanced by Rac1(Q61L) and effectively inhibited by the Rac binding site mutation (R103E). Data are from at least three independent transfection experiments. E. Transfected Noxa1 proteins are detected by Western blotting at comparable levels. Similar protein loading is confirmed by β-tubulin blotting.

basal activity in the transfected cell model but little effect on PMA-stimulated activity. Wild-type Rac1 shows no statistically significant enhancing effect. Overexpression of a protein encoding the Cdc42/Rac-interactive binding (CRIB; aa 66 to 147) region of human p21-activated kinase 2 (hPAK2) shows a weaker inhibitory effect (10 to 15% inhibition) than that of Rac1(G30S) in CHO-K1 cells but not in HEK293 cells (data not shown). The absolute levels of Nox1 activity reconstituted by Noxo1 and Noxa1 in CHO-K1 cells are ~10% of that in HEK293 cells (data not shown). Interestingly, CHO-K1 cells show higher enhancing effects by PMA stimulation on oxidase activity than those observed in HEK293 cells (Fig. 3A and B).

To clarify the basis for the discrepancies in the effects of Rac1 mutants on Nox1 activity in the two reconstituted models, we examined the effects of Rac1 mutants on the reconstituted Nox2 system, which is known to involve Rac. Surprisingly, the Nox2 system transfected into HEK293 cells is not inhibited by the "dominant-negative" mutant Rac1(T17N), while this mutant does inhibit Nox2 activity when transfected into CHO-K1 cells (Fig. 3D). The effector site mutant Rac1 (G30S) is an effective inhibitor of Nox2 in both cell models. The greater effectiveness of the Rac mutants in CHO-K1 cells may, in part, relate to higher expression of the transfected Myc-tagged proteins relative to endogenous Rac1 levels (Fig. 3D). Experiments examining the subcellular location of Rac1 in these cells, however, reveal other differences between the two models (Fig. 3E). In HEK293 cells, a significant amount of endogenous Rac1 is concentrated along the plasma membrane within cortical regions of the cell, regardless of cellular stimulation. In contrast, endogenous Rac1 in CHO-K1 cells is detected primarily in a diffuse granular pattern throughout the cytoplasm (Fig. 3E), with small amounts of Rac1 seen in ruffling membranes (data not shown). Finally, a Rac1 activation assay using the CRIB region of hPAK1 detected active Rac1 in unstimulated HEK293 cells but not in CHO-K1 cells (Fig. 3F). Together, these observations could explain how overexpressed Rac1 mutants are less capable of perturbing endogenous Rac1 pools and altering oxidase activity (Nox1 or Nox2) in the HEK293 cell model.

To explore further the involvement of Rac1 in the complete Nox1 system, we used a vector-based method for expressing shRNA targeted to Rac1 in HEK293 cells. pSUPER-Rac1-147,

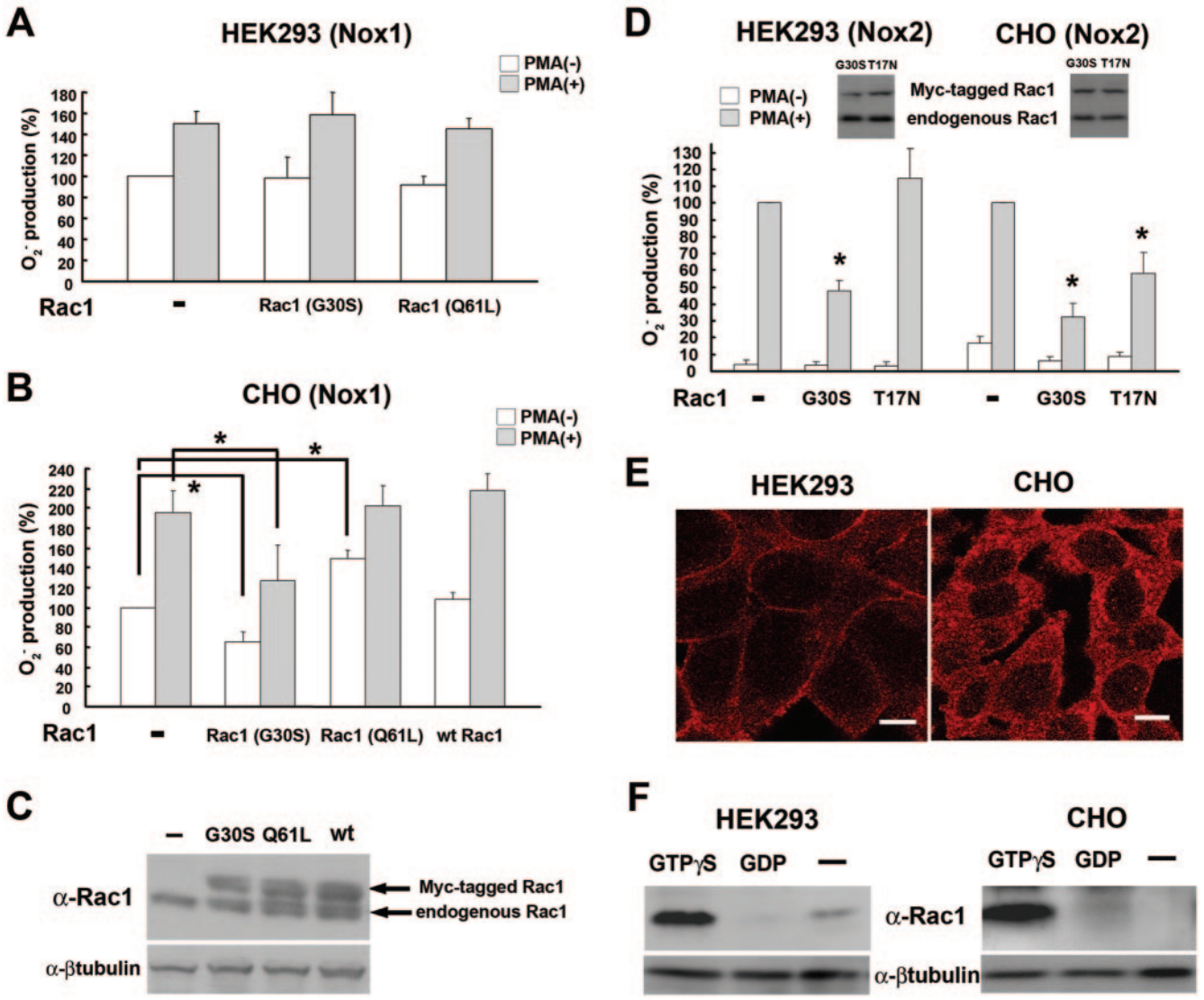

FIG. 3. Rac1 involvement in the fully activated Nox1 system. A. Neither Rac1(G30S) nor Rac1(Q61L) affects Nox1 activity in the HEK293 cell model. Data are from at least three independent transfection experiments. B. Rac1(G30S) inhibits both basal and PMA-stimulated Nox1 activity in transfected CHO-K1 cells, while Rac1(Q61L) enhances basal activity. Data represent means ± SD of at least three independent transfection experiments. (*, $P < 0.05$, compared with no Rac1 transfection [pcDNA3.1]). C. Western blotting of CHO-K1 cell lysates detects both the endogenous and Myc-tagged Rac1 proteins. Comparable protein loading is confirmed by β-tubulin blotting. D. Rac1(G30S) inhibits Nox2 activity in transfected HEK293 and CHO-K1 cells, while Rac1(T17N) inhibits Nox2 only in CHO-K1 cells. Data represent means ± SD of at least three independent transfection experiments. (*, $P < 0.05$, compared with no Rac1 transfection [pcDNA3.1]). Western blotting (top panels) detects endogenous and the mutant Myc-tagged Rac1 proteins. E. Immunofluorescence detection of endogenous Rac1 in HEK293 cells reveals significant Rac1 accumulation along the plasma membrane, while Rac1 is localized primarily to the cytosol in CHO-K1 cells. Bar, 10 μm. F. Rac1 activation assay detects activated endogenous Rac1 (GTP bound) in unstimulated HEK293 cells but not in CHO-K1 cells. Similar results were obtained in two independent experiments.

pSUPER-Rac1-681, and pSUPER-Rac1-1664 significantly reduce detectable levels of endogenous Rac1 protein, while pSUPER-Rac1-320 has no apparent effect (Fig. 4A). These observations correlate with significant inhibition of Nox1 activity by the effective Rac1-targeting vectors (Fig. 4B). In particular, the most potent Rac1-suppressing vector, pSUPER-Rac1-681, strongly inhibits ROS production reconstituted by Nox1 and Noxa1(pp) (12.9% ± 3.3% of control activity) (Fig. 4B), a result consistent with data showing that Nox1 activity supported by Noxa1(pp) is completely abolished by Noxa1(pp, R103E) (Fig. 2C). In addition, pSUPER-Rac1-681 significantly reduces the activity of Nox1 supported by Noxo1 and Noxa1 together (42.6% ± 4.8% of control). These results suggest that Rac1 supports the fully reconstituted Nox1 system and indicate that, in the absence of Noxo1, the membrane-targeted form of Noxa1 [Noxa1(pp)] is even more dependent on Rac1, since Noxa1(pp) does not support significant ROS production when endogenous Rac1 levels are suppressed. Thus, it appears that activation of both the Nox1 and Nox2 systems requires not only membrane targeting of the Nox activators (Noxa1 or $p67^{phox}$) but also interaction with Rac.

The inhibition of Nox1 activity by Rac1 silencing was also confirmed by the dose-dependent effects of synthetic siRNA

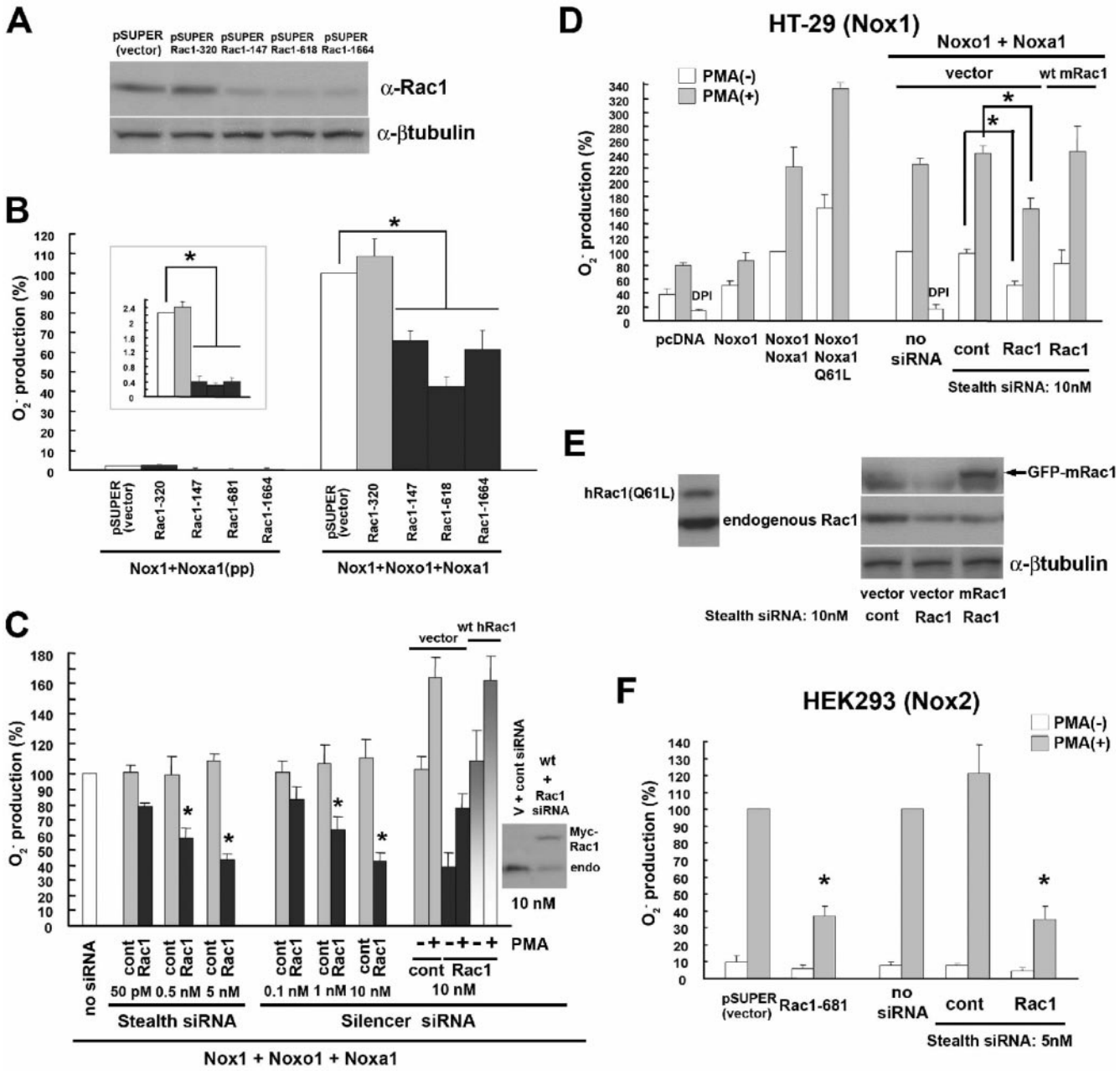

FIG. 4. Suppression of Nox1 activity by Rac1-specific siRNA. A. Transfection with pSUPER-Rac1-147, pSUPER-Rac1-681, or pSUPER-Rac1-1664 but not pSUPER Rac1-320 results in significant knockdown of endogenous Rac1 protein levels compared with control plasmid lacking RNAi sequence [pSUPER (vector)] in HEK293 cells. Similar protein loading is confirmed by β-tubulin blotting. B. Unstimulated Nox1 activity supported by Noxa1(pp) is significantly inhibited by pSUPER-Rac1-147, pSUPER-Rac1-681, or pSUPER-Rac1-1664 in HEK293 cells. Data represent means ± SD of at least three independent transfection experiments [∗, $P < 0.05$, compared with pSUPER (vector)]. C. Unstimulated Nox1 activity supported by Noxo1 and Noxa1 is also inhibited by Rac1 Stealth siRNA or Rac1 Silencer siRNA in HEK293 cells, and reintroduction of wild-type human Rac1 (0.5 μg) restores Nox1 activity suppressed by Rac1 Silencer siRNA (both unstimulated and PMA stimulated). Right, Western blotting confirms significant suppression of endogenous Rac1 protein by Rac1 Silencer siRNA and reintroduction of Myc-tagged wild-type human Rac1 (lysates are from the same number of cells). Data represent means ± SD of at least three independent transfection experiments (∗, $P < 0.05$, compared with no siRNA and negative-control siRNA). D. Cotransfection of Noxo1 and Noxa1 enhances basal and PMA-stimulated ROS production by HT-29 cells. These activities are further enhanced by Rac1(Q61L). ROS production supported by Noxo1 and Noxa1 is significantly inhibited by Rac1 Validated Stealth siRNA and is restored by reintroduction of wild-type mouse Rac1. Data represent means ± SD of at least three independent transfection experiments (∗, $P < 0.05$, compared with negative-control siRNA). E. Western blotting of HT-29 cell lysates confirms expression of Rac1(Q61L), suppression of endogenous Rac1 protein by Rac1 Stealth siRNA, and reintroduction of GFP-tagged wild-type mouse Rac1 (lysates are from the same number of cells, and similar protein loading is further confirmed by β-tubulin blotting). F. Transfected vector-based RNAi (pSUPER-Rac1-681) and synthetic Rac1 siRNA (Stealth siRNA) are also effective inhibitors of the Nox2 system in HEK293 cells. Data represent means ± SD of at least three independent transfection experiments [∗, $P < 0.05$, compared with no siRNA and negative-control siRNA or pSUPER (vector)].

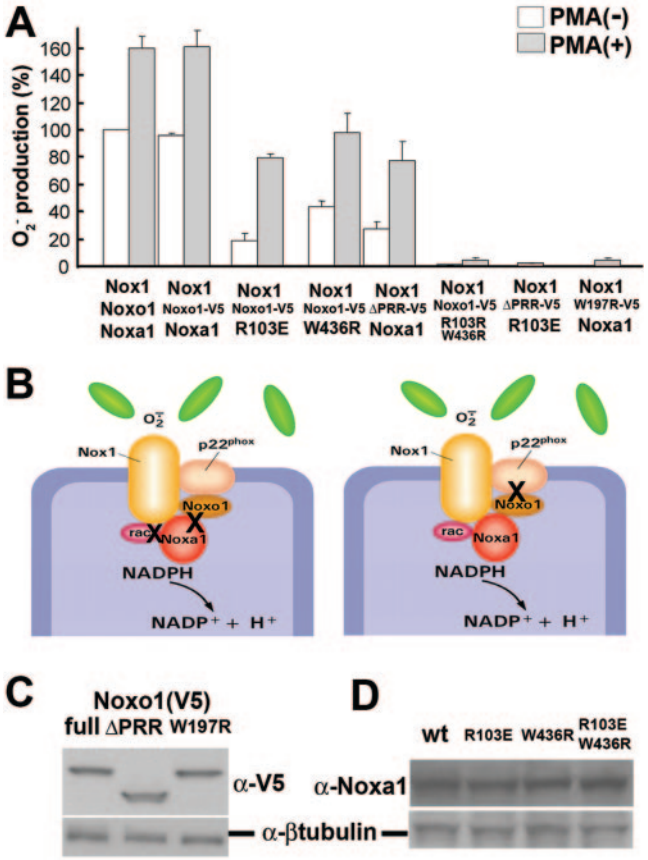

FIG. 5. Disruption of the fully activated Nox1 system by mutations affecting interactions with Rac1, Noxo1, Noxa1, and p22*phox*. A. The fully activated Nox1 system, supported by Noxo1 and Noxa1, is only partially affected by Noxa1(R103E), Noxa1(W436R), or Noxo1(ΔPRR) mutations, which disrupt interactions with Rac1, Noxo1. or Noxa1, respectively. Combined disruption of Noxa1-Rac1 and Noxo1-Noxa1 interactions completely inhibits the Nox1 system, as does a disruption of the Noxo1-p22*phox* interaction. Data are from at least three independent transfection experiments. B. Schematic representation of sites that effectively disrupt Nox1 activity, involving mutations in Noxa1 (left) or Noxo1 (right). The panel is adapted from reference 22. C. Western blotting of HEK293 cell lysates confirms comparable expression of Noxo1-V5 mutants. D. Western blotting confirms comparable expression of Noxa1 mutants. Lower (control) panels confirm comparable protein loading by β-tubulin blotting.

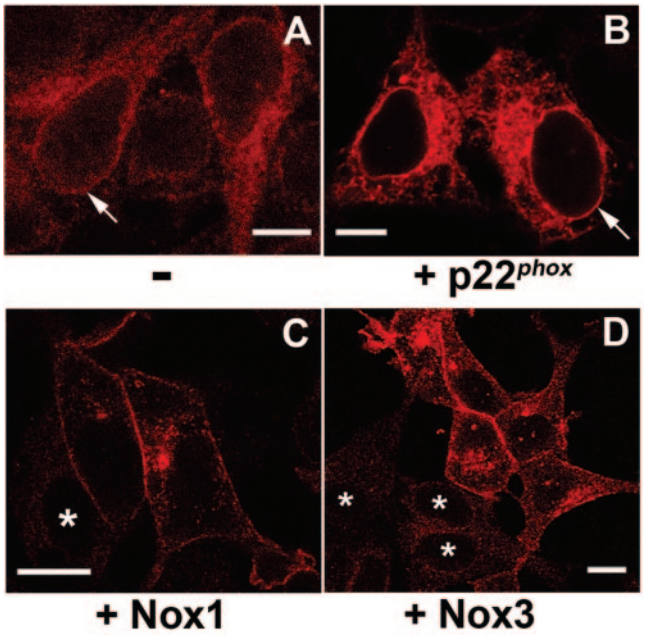

FIG. 6. Nox1- and Nox3-dependent targeting of p22*phox* to the plasma membrane. A and B. Immunofluorescence imaging of endogenous (A) and transfected (B) p22*phox* in HEK293 cells, showing reticular cytosolic and nuclear membrane (arrows) staining patterns. C. Transfection of Nox1 results in a redistribution of endogenous p22*phox* to the plasma membrane. D. A similar redistribution of p22*phox* occurs in Nox3-transfected cells. *, untransfected cells showing primarily cytosolic staining patterns and overall weaker staining.

transfection, where the complete Nox1 system is significantly inhibited (44.2% ± 4.2% or 41.8% ± 5.4% of control values) by Rac1 Validated Stealth siRNA or Rac1 Pre-designed Silencer siRNA, respectively (Fig. 4C). The specificity of these Rac1 silencing effects was demonstrated by complete restoration of Nox1 activity inhibited by Rac1 Silencer siRNA (targeted to the 3′ untranslated region of mRNA of human Rac1) following transfection of wild-type human Rac1 cDNA (Fig. 4C). To demonstrate involvement of Rac1 in oxidase activity of colon epithelial cells, we examined the effects of mutant Rac1 and Rac1 siRNA in HT-29 cells, which express endogenous Nox1 (19, 20). Cotransfection of these cells with Noxo1 and Noxa1 significantly enhances basal ROS production, which is inhibited by DPI (Fig. 4D). This activity is enhanced further by Rac1(Q61L) (Fig. 4D). Furthermore, ROS production enhanced by Noxo1 and Noxa1 is significantly inhibited by Rac1 Validated Stealth siRNA (targeted to the coding region of mRNA of human Rac1) and is restored by reintroduction of wild-type mouse Rac1, which differs within the targeted human sequence by 4 nucleotides (Fig. 4D). Finally, we validated our Rac1 silencing approach used in these experiments by demonstrating that both the specific vector-based and synthetic siRNAs are also effective inhibitors of the Nox2 system reconstituted in the HEK293 cell model (Fig. 4F). These observations on the involvement of Rac1 in Nox1 activity in the three transfected models confirm and extend the observations of Kawahara et al., who showed restoration of Ly294002-inhibited ROS production in guinea pig gastric pit cells by overexpressing an active mutant of Rac1 (28).

Nox1 is tightly controlled by multiple interactions involving Noxo1, Noxa1, Rac1, and p22*phox*. We then studied the assembly and activation of the entire Nox1 system by examining the effects of disrupting various protein-protein interactions within this complex (Fig. 5A). Basal Nox1 activity, reconstituted by Noxo1 and Noxa1, was reduced when the Rac1-binding mutant, Noxa1(R103E), was substituted for wild-type Noxa1, although the activity was still PMA stimulated. Also, the replacement of Noxa1 with the defective Noxo1-binding mutant, Noxa1(W436R), reduces the activity of Nox1, and the activity is still PMA stimulated. The effect of this mutant was confirmed further by expression of the complementary, C-terminal deletion mutant of Noxo1, Noxo1(ΔPRR). However, Nox1 activity supported by Noxo1 and Noxa1 was effectively abolished by the combined Noxa1 mutant Noxa1(R103E, W436R), by the complementary mutants Noxo1(ΔPRR) and Noxa1(R103E),

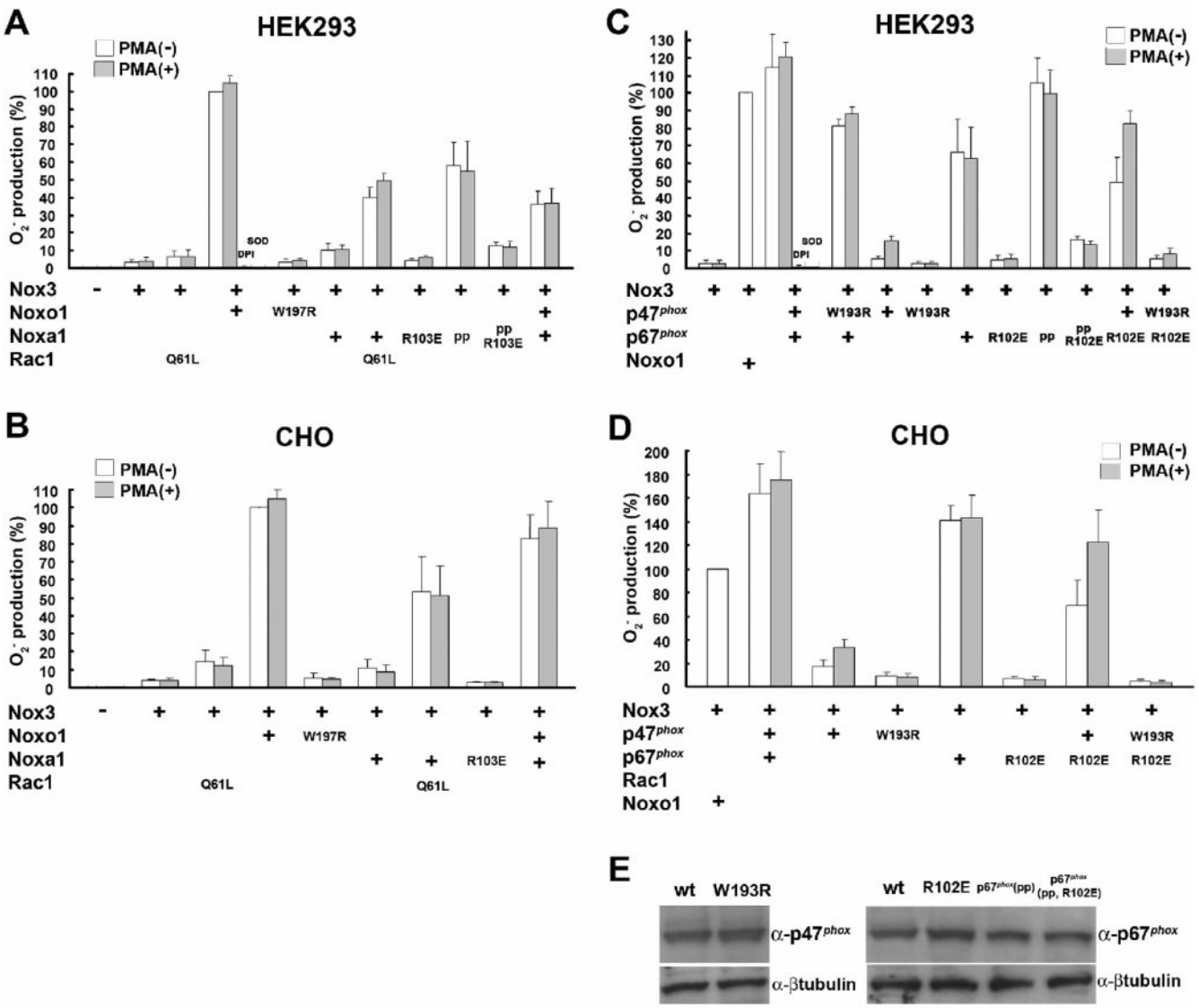

FIG. 7. Nox3 activity supported by Noxa1 or p67^{phox} is Rac1 dependent. A and B. Reconstitution of Nox3 activity with Nox proteins (Noxo1 or Noxa1) in HEK293 and CHO-K1 cells. Noxo1-supported activity is abolished by the SH3 domain (p22^{phox} binding site) mutation. Noxa1-supported activity is enhanced by Rac1(Q61L) and abolished by the Rac1 binding site mutation (R103E). Nox3 activity supported by membrane-targeted Noxa1 is inhibited by the R103E mutation. Data are from at least three independent transfection experiments. C and D. Reconstitution of Nox3 activity with phox proteins in HEK293 and CHO-K1 cells. The p47^{phox}-supported Nox3 activity is inhibited by the p22^{phox} binding site mutation (W193R), while Nox3 activity supported by p67^{phox} (wild-type and membrane-targeted forms) is inhibited by the Rac1 binding site mutation. Data are from at least three independent transfection experiments. E. Western blotting of HEK293 cell lysates detects comparable levels of p47^{phox} (left) and p67^{phox} (right) mutant proteins. Comparable protein loading is confirmed by β-tubulin blotting.

or by the substitution of Noxo1(W197R) for Noxo1. Noxo1 (W197R) is the homologous point mutant of p47^{phox}(W193R) that disrupts the interaction between the SH3 domain of p47^{phox} and its PRR target site in p22^{phox} (17, 51). Thus, the Nox1 system is effectively inhibited either (i) by disruption of the interactions of Noxa1 with both of its partners, Noxo1 and Rac1, thereby blocking access of Noxa1 to Nox1; or (ii) by disruption of the interaction between Noxo1 and p22^{phox}, thereby preventing the Noxo1-Noxa1 complex from interacting with the Nox1-p22^{phox} complex on the plasma membrane (Fig. 5B). Together, these results demonstrate that full activation of the Nox1 system involves interactions between multiple components (Nox1, Noxo1, Noxa1, Rac1, and p22^{phox}) that are functionally analogous to all of the critical components of the phagocytic (Nox2) system.

To demonstrate further p22^{phox} involvement in the assembly of the Nox1 system, we examined the subcellular localization of p22^{phox} in transfected cells. Endogenous p22^{phox} detected in HEK293 cells by confocal immunofluorescence microscopy has a reticular intracellular and perinuclear staining pattern (Fig. 6A), and transfected p22^{phox} displays a staining pattern similar to that of endogenous p22^{phox} (Fig. 6B). Interestingly, Nox1 cotransfection results in dramatic rearrangements of the endogenous p22^{phox} staining pattern, leading to significant ac-

cumulation of p22^{phox} on the plasma membrane (Fig. 6C). These observations support the idea that Nox1 is stabilized through its association with p22^{phox} and that the two chains are transported together to the plasma membrane, where they provide a docking site for the Noxo1-Noxa1 complex. Thus, the activated enzyme would release ROS extracellularly, as is evident from the efficient scavenging effects of exogenously added SOD (Fig. 2A).

Rac1 is involved in the Nox3 system. Nox3 also appears to function as a multicomponent system involving p22^{phox} and either the Nox or phox supportive cofactors (8, 15, 55). We examined assembly and activation mechanisms of the Nox3 system with a focus on potential involvement of Rac1. As shown in Fig. 6D, transfection of Nox3 in HEK293 cells results in p22^{phox} transport to the plasma membrane, as observed with Nox1. Taken together with results showing that Nox3 activity is completely abolished by exogenous SOD (Fig. 7A and B), the Nox3 system appears to release ROS into the extracellular medium, like the Nox1 system.

The physiologically relevant partners of Nox3 have not been clarified; therefore, we examined Nox3 activation using both Nox and phox supportive cofactors in both transfected cell models (Fig. 7). Consistent with previous work (8, 15, 55), transfected Nox3 exhibits detectable activity in the absence of any cotransfected organizer or activator protein. Interestingly, the activity of Nox3 alone was enhanced by Rac1(Q61L). Nox3 activity is maximally enhanced by cotransfection of Noxo1 alone and thus was used as a reference for comparing activities of all other Nox3 partners tested in the HEK293 and CHO-K1 cell models (Fig. 7A and B). This activity is effectively abolished by substitution of Noxo1(W197R) for Noxo1, suggesting that the Noxo1-p22^{phox} interaction is critical for Nox3 activity supported by Noxo1. The combined expression of Noxa1 along with Noxo1 results in lower Nox3 activity than that supported by Noxo1 alone. This is consistent with recent findings by others (55), suggesting that Noxo1 and Noxa1 together are not optimally suited as partners of Nox3 or act together to downregulate Nox3. Noxa1 alone supported low levels of Nox3 activity, but Rac1(Q61L) enhanced this activity. Nox3 activity supported by Noxa1(pp) was higher than that supported by Noxa1 or Noxa1 and Rac1(Q61L). The Nox3 activity supported by Noxa1 or Noxa1(pp) is dramatically decreased by the defective Rac-binding mutants (R103E) of these proteins. Thus, Nox3 activity appears to involve participation of both Rac1 and p22^{phox} interactions, although Nox3 is not subject to the same strict requirements for both Nox organizer and activator proteins that are observed with Nox1.

The Nox3 activity supported by phox regulators also appears to involve both p22^{phox} and Rac1 interactions. Surprisingly, the activities of Nox3 supported by p47^{phox} and p67^{phox} together are not significantly responsive to PMA, in both the transfected HEK293 and CHO-K1 cell models (Fig. 7C and D). These are higher than those supported by Noxo1 alone. The activity of these systems is not dramatically inhibited by expression of the p47^{phox}(W193R) mutant, which disrupts the interaction between the SH3 domain of p47^{phox} and its PRR target site in p22^{phox} (17, 51). Nox3 is supported at low levels by p47^{phox} alone in both cell models; this activity is enhanced by PMA and abolished by replacement with the p47^{phox}(W193R) mutant. Nox3 is activated to a significant extent by p67^{phox} alone, and

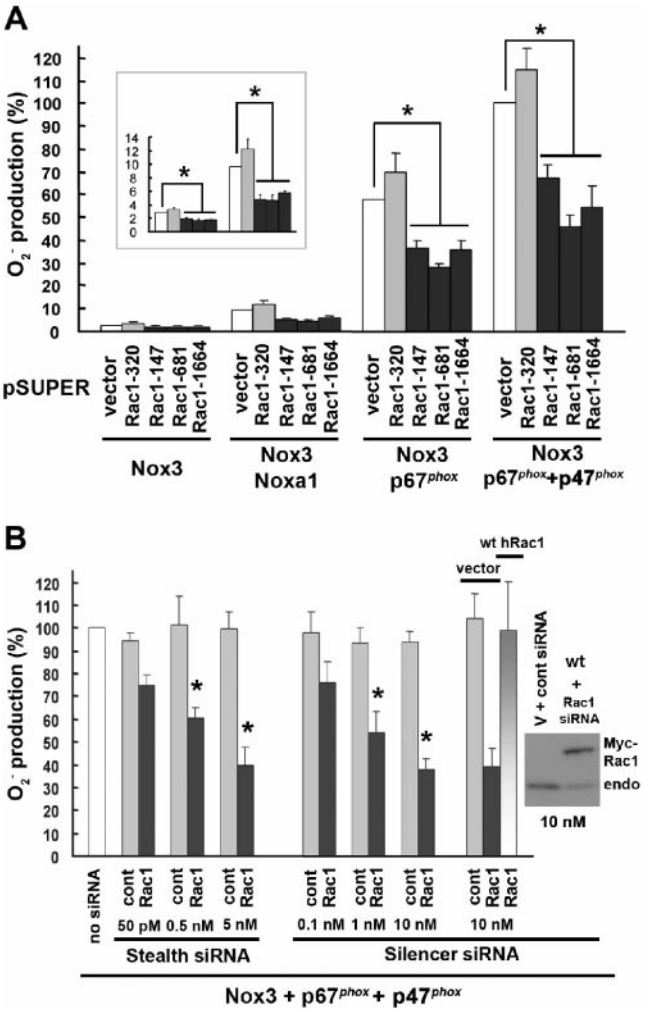

FIG. 8. Suppression of Nox3 activity by Rac1-specific siRNAs in HEK293 cells. A. Nox3 basal activity, as well as Nox3 activity supported by Noxa1, p67^{phox}, or p67^{phox} and p47^{phox}, is significantly inhibited by pSUPER-Rac1-147, pSUPER-Rac1-681, or pSUPER-Rac1-1664 (vector-based RNAi). Data represent means ± SD of at least three independent transfection experiments [*, $P < 0.05$, compared with pSUPER (vector)]. B. Nox3 activity supported by p67^{phox} and p47^{phox} is significantly inhibited by Rac1 Stealth siRNA or Rac1 Silencer siRNA and restored by reintroduction of wild-type human Rac1 (0.5 μg). Right panel, Western blotting confirms significant knockdown of endogenous Rac1 protein by Rac1 Silencer siRNA and production of Myc-tagged wild-type human Rac1 (lysates are from the same number of cells). Data represent means ± SD of at least three independent transfection experiments (*, $P < 0.05$, compared with no siRNA and negative-control siRNA).

the activity supported by p67^{phox}(pp) alone reaches almost the same level as that of the Nox3-p47^{phox}-p67^{phox} system. Together, these observations suggest that p47^{phox} plays a relatively minor role in supporting Nox3, in comparison with p67^{phox}. Nox3 activity supported by p67^{phox} or p67^{phox}(pp) is dramatically reduced by p67^{phox}(R102E) or p67^{phox}(pp, R102E). Residue Arg102 in p67^{phox} forms direct hydrogen bonds with Rac1, and the mutation to Glu disrupts the ability of p67^{phox} to support the Nox2(phox)-based oxidase (33, 37). Expression of Rac1(Q61L) does not result in any further en-

hancing effects on p67^{phox} or p67^{phox}(pp)-supported Nox3 activity (data not shown). Finally, the activity of Nox3 supported by p47^{phox} and p67^{phox} is partially inhibited by replacement with the p67^{phox}(R102E) mutant and almost completely abolished by coexpression of p47^{phox}(W193R) and p67^{phox}(R102E). Together, these results provide strong evidence for the involvement of Rac1 in the Nox3 system reconstituted with phox regulators in both transfected models.

To demonstrate further the involvement of Rac1 in the Nox3 system, we used the RNAi method to suppress Rac1 levels in the HEK293 cell model (Fig. 8). Basal Nox3 activity is reduced to 60.2% ± 9.2% of control levels with Rac1-specific siRNA (pSUPER-Rac1-681). Nox3 activity reconstituted by Noxa1 alone or by p67^{phox} alone is reduced to 48.2% ± 8.0% or 48.6% ± 3.8%, respectively, with pSUPER-Rac1-681. Finally, the full activity of Nox3 reconstituted by p47^{phox} and p67^{phox} together is also significantly reduced to 45.6% ± 5.6% by pSUPER-Rac1-681 (Fig. 8A). These results were confirmed by synthetic siRNA transfection experiments, in which Nox3 activity supported by p47^{phox} and p67^{phox} is inhibited more than 60% by two different reagents (39.9% ± 8.2% or 37.8% ± 5.0% by Rac1 Validated Stealth siRNA or Rac1 Pre-designed Silencer siRNA, respectively) (Fig. 8B). Moreover, the activity inhibited by Silencer siRNA is completely restored by reintroduction of wild-type human Rac1 (Fig. 8B).

To examine in more detail the basis of p67^{phox}-supported Nox3 activity, we explored the subcellular localization of p67^{phox}. As observed with Noxa1, p67^{phox} localizes to membrane ruffles (Fig. 9A), with an appearance that closely resembles that of Rac1, consistent with recent observations in endothelial cells (11); however, this colocalization with Rac1 is not disrupted with Noxa1(R103E) (Fig. 9B). These observations were confirmed in cellular fractionation experiments, which do not detect any changes in p67^{phox} distribution between membrane and cytosolic fractions in cells expressing the defective Rac-binding mutant form p67^{phox}(R102E) (Fig. 9C, left) or in cells with suppressed Rac1 expression caused by siRNA (Fig. 9C, right). In contrast, p67^{phox}(pp) is localized predominately on the plasma membrane (Fig. 9E), consistent with its ability to support higher levels of Nox3 activity in comparison with wild-type p67^{phox} (Fig. 7C). These observations suggest that Rac1 does not serve as a determinant in the subcellular localization of p67^{phox}.

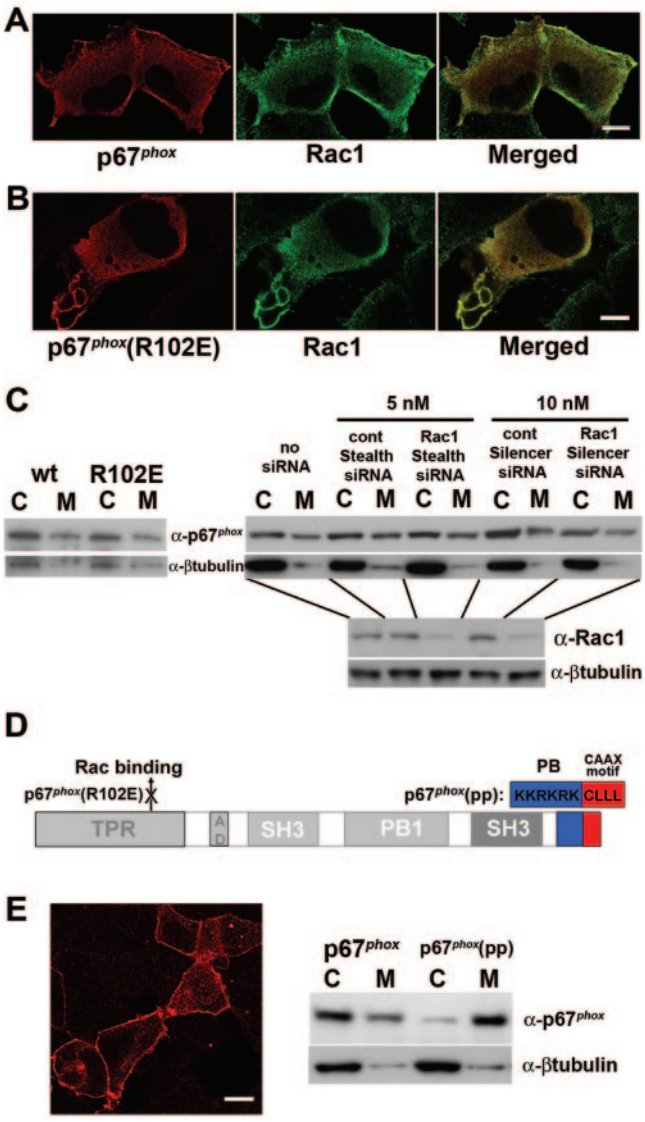

FIG. 9. p67^{phox} localizes to ruffling membranes in HEK293 cells. A. p67^{phox} is detected in cytosol and ruffling membranes; Rac1 colocalizes with p67^{phox} on membrane ruffles. B. p67^{phox}(R102E) also colocalizes with Rac1 within ruffling membranes. C. Wild-type and p67^{phox}(R103E) are distributed similarly between cytosol and membrane fractions. Right, fractionation of p67^{phox} is not affected by two Rac1-specific siRNAs. Immunoblotting of total lysates shows effective knockdown of endogenous Rac1 by the Rac1-specific siRNAs. no siRNA, transfection without siRNA. D. Schematic representation of the p67^{phox}(pp) structure. E. Plasma membrane targeting of p67^{phox}(pp). Right, fractionation study confirms p67^{phox}(pp) targeting to the membrane fraction. Bar, 10 μm. Comparable protein loading is confirmed by β-tubulin blotting. Similar results were obtained in four (A, B, C [left], and E [left]), three (C [right]), or two (E [right]) separate experiments.

DISCUSSION

In this study, we explored determinants of cellular targeting, assembly, and activation of the two closest homologs of the phagocytic (Nox2-based) NADPH oxidase, Nox1 and Nox3, and obtained evidence indicating that both of these multicomponent enzymes are regulated by the small GTPase Rac1. Both enzymes are supported by "activator" and "organizer" components, although their dependence on these regulators differs significantly (7, 8, 15, 21, 54, 55). By systematically expressing wild-type and mutant forms of these components in transfected cell models, we assessed the relative contributions of these regulators and delineated a hierarchy of interactions governing assembly and activation of these novel oxidase complexes.

The molecular mechanisms controlling phagocytic oxidase activity have been studied extensively (6, 40, 43, 53). The importance of multiple, specific protein-protein interactions in the assembly of this complex has been appreciated through analysis of a variety of molecular lesions observed in chronic granulomatous disease. For example, both flavocytochrome subunits are needed to stabilize this protein, enable complete posttranslational processing, and direct the mature protein to the plasma membrane (47, 58, 59). The p47^{phox} "adaptor" component binds to membrane lipids, is tethered to the cyto-

chrome b_{558} through direct interactions with p22^{phox}, and is involved in linking other cytosolic phox proteins to this complex (41, 52). Rac and p67^{phox} together have more direct roles in regulating electron flow through the flavocytochrome through GTP-dependent interactions (10). The phagocytic enzyme is capable of robust oxidative output, although its activity is effectively suppressed in resting cells as the components of the latent enzyme are maintained in separate compartments. The flavocytochrome is stored within intracellular membranes (27), Rac is maintained in a GDP-bound cytosolic complex dimerized with Rho-GDI (9), and the other phox proteins associate in a separate cytosolic complex in a dephosphorylated state (6, 40, 43). Following cellular activation, these components assemble into the active, membrane-bound complex through distinct, but coordinated, signaling events. The flavocytochrome translocates to specific membrane domains; the cytosolic components are phosphorylated, thereby inducing conformational changes that favor interactions with the cytochrome b_{558}; and Rac dissociates from RhoGDI and translocates independently to the membrane following exchange of GDP for GTP (26, 60). Extensive studies of cell-free oxidase reconstitution suggest that Rac can serve two functions in supporting the Nox2 complex: tethering p67^{phox} to the membrane and inducing conformational changes in p67^{phox} that promote oxidase activation (3, 23, 24, 49). Our current observations in transfected cell models indicate that Rac1 also functions in supporting Nox1- and Nox3-based oxidase systems through interactions with the "activator" components; however, we did not obtain evidence supporting the proposal that Rac1 serves as a carrier protein that directs the subcellular location of these activators (Fig. 1F and G and Fig. 9C).

Our studies, together with other recent findings, indicate that a similar hierarchy of interactions occurs in the reconstituted Nox1 system. Nox1 and p22^{phox} appear to associate and function as a heterodimeric complex, in that the coexpression of both chains leads to enhanced oxidative output and increased stabilization of both subunits (4, 30, 54). These chains form a physical complex, as assessed by colocalization, coimmunoprecipitation, and fluorescence resonance energy transfer (4, 25, 30). We have now shown that Nox1 coexpression enables specific targeting of p22^{phox} to the plasma membrane (Fig. 6), along with release of ROS into the extracellular compartment. Noxa1 is also targeted to the plasma membrane through tail-to-tail interactions with Noxo1 (Fig. 1), analogous to the functional relationship between p67^{phox} and p47^{phox}. However, Noxo1 membrane interactions do not play the same critical role as p47^{phox} in regulating assembly, since Noxo1 is detected on the membrane even in the absence of cellular activation (14). The association of Noxo1 with the membrane was attributed primarily to constitutive PX domain-membrane lipid interactions (14), which occur in the absence of sequence homologous to the phosphorylated autoinhibitory domain of p47^{phox}. Nonetheless, we and others demonstrated the importance of the Noxo1-p22^{phox} interaction through the inhibitory effects of mutations that block this interaction on Nox1 activity (Fig. 5) (30, 54). Noxo1 participation in the Nox1 system appears to be dispensable when Noxa1 is adapted with sequence that can otherwise target this protein to the membrane, since this form of Noxa1 [Noxa1(pp)] can effectively support oxidase activity in the absence of Noxo1. Previous cell-free reconstitution studies have shown that the p47^{phox} component is also dispensable in the Nox2 system, when p67^{phox} and Rac are provided in excess (18, 34) or when fusion proteins of p67^{phox} are constructed that anchor p67^{phox} to membrane (3, 23, 24, 42). Thus, like p47^{phox}, Noxo1 appears to function principally as an adaptor that bridges interactions, in this case between Noxa1 and the membrane (lipid and p22^{phox}). Finally, Noxa1 functions as a Rac1-GTP-dependent modulator of Nox1 activity, analogous to the role of p67^{phox} in Rac-dependent regulation of Nox2 activity (3, 10, 23, 24, 42).

We obtained three independent lines of evidence indicating involvement of Rac1 in Nox1 regulation: (i) mutant forms of Noxa1 defective in Rac1 binding support lower levels of Nox1 activity than do wild-type Noxa1, (ii) overexpressed mutant forms of Rac1 affect Nox1 activity, and (iii) siRNA-mediated suppression of Rac1 expression results in diminished Nox1 activity, which is restored by wild-type Rac1 overexpression. The participation of Noxa1 and Rac1 as GTP-dependent interacting regulators of Nox1 activity was most evident in the absence of Noxo1. This was particularly clear when the requirement for Noxo1 was partially compensated by Noxa1(pp) (Fig. 2 and 4), which localizes at the plasma membrane but does not support ROS production without interaction with Rac1. Our observations on the Rac1-GTP dependence of Noxa1(pp) (Fig. 2 and 4) are analogous to those of Alloul et al. and Gorzalczany et al. on a p67^{phox}-Rac1 chimera protein, p67^{phox} (1-212)-Rac1 (178-192), which binds to the membrane but is unable to support Nox2 activity in vitro in the absence of the Rac1-GTP moiety that interacts with p67^{phox}; addition of Rac1-GTPγS or insertion of Rac1 effector moiety to the chimera p67^{phox} (1-212)-Rac1 (1-192) supports high Nox2 activity even in the absence of p47^{phox} (3, 23). Analysis of the effects of multiple disrupted interactions (Fig. 5A and B), as well as the effects of Rac1-targeting siRNAs, indicated Rac1 involvement even with the fully reconstituted Nox1 system. Our results implicating Rac1 in Nox1 activation are further supported by recent work suggesting Rac1 participation in lipopolysaccharide-mediated Nox1 activation in gastric pit cells (28), as well as work suggesting involvement of the Rac1 guanine nucleotide exchange factor beta Pix in Nox1 activation (46), although these studies did not examine the involvement of Noxa1 as the direct effector of Rac1. It is not entirely clear which Nox1 component accounts for PMA-enhanced oxidase activity, although Noxo1 is the least likely candidate. Unlike p47^{phox}, Noxo1 is membrane bound even without cell stimulation, does not contain protein kinase C phosphorylation sites, and does not show PMA-dependent changes in cellular localization (data not shown). Furthermore, PMA-stimulated activity is observed in the absence of Noxo1, suggesting that Noxa1, Rac1, or other upstream regulatory components are subject to PMA stimulation.

The effectiveness of Rac1 overexpression on Nox1 or Nox2 activity is dependent on the host cell line tested and the type of Rac1 mutant expressed. Mutant forms of Rac1 have little apparent effect on the fully reconstituted Nox1 system in HEK293 cells, although the same approach provided evidence for Rac1 involvement in the CHO-K1 cell model. HEK293 cells show significant accumulation of Rac1 in cortical regions and along the plasma membrane, even without stimulation, while CHO-K1 cells have a dispersed, but granular, cytoplas-

mic Rac1 staining pattern and exhibit a higher PMA-stimulated oxidase component. Thus, the effects of the overexpressed Rac1 mutants on the available Rac1 pools regulating oxidase activity may differ in the two cell lines. The Rac1(T17N) mutant does not affect Nox1 activity in any transfected cell line (data not shown), although Rac1(T17N) suppressed Nox2 activity in CHO-K1 cells but not in HEK293 cells (Fig. 3E). Overexpressed Rac1(G30S) is effective in suppressing both Nox1 (CHO-K1 cells) and Nox2 (HEK293 and CHO-K1 cells); moreover, Rac1(G30S) more effectively suppressed Nox2 activity than Rac1(T17N) in CHO-K1 cells (Fig. 3B and E). Previous work showed that Rac1(G30S) does not support the activity of the Nox2 complex in vitro (35, 49), and crystallographic data indicate that this residue forms direct hydrogen bonds with its effector, p67^{phox} (37). Thus, Rac1(G30S) is a more effective "dominant-negative" mutant affecting Nox1 or Nox2 complexes than Rac1(T17N).

In agreement with recent reports (8, 15, 55), we found that Nox3 is distinctly different from Nox1 and Nox2, as it is more flexible in its requirements for classical (phox) or novel (Nox) supportive cofactors. Nox3 exhibits significant constitutive activity when expressed alone, but its activity is enhanced further in the presence of one or more supportive cofactors. We observed that Nox3 exhibits the following order of preference for modulators: p47^{phox} plus p67^{phox} > Noxo1 > p67^{phox} > p47^{phox} > Noxa1. Cheng et al. reported that Noxa1 slightly enhances Nox3 activity supported by Noxo1 (15), although we observed that Noxa1 inhibits Nox3 activity supported by Noxo1, consistent with observations by Ueno et al. in HEK293, CHO-K1, or Cos-7 cell models (55). Thus, Nox3 does not exhibit strict requirements for both an "activator" and "organizer" components. We have shown through mutagenesis of the "organizer" components that their ability to support Nox3 likely involves their interaction with p22^{phox} and that Nox3 enables its transport to the plasma membrane. While the manuscript was in preparation, similar conclusions about the dependence of Nox3 on p22^{phox} were described (55), based on the effects of p22^{phox}, p47^{phox}, and Noxo1 mutagenesis on Nox3 activity. However, Ueno et al. (55) concluded that Rac1 is not a regulator of Nox3, contrary to our current observations indicating that Rac1 regulates Nox3 activity.

Our evidence for Rac1 involvement in Nox3 activity includes the following: (i) Nox3 activity supported by p67^{phox} or Noxa1 (whether wild type or the membrane-targeted forms) is inhibited by mutations in the Rac1 binding sites of these proteins; (ii) Nox3 activity alone, or that supported by Noxa1, is enhanced by coexpression of constitutively active Rac1(Q61L); (iii) siRNA-mediated suppression of cellular Rac1 production (with five different Rac1-targeted siRNAs) results in lower Nox3 activity when expressed in the contexts of the presence of p47^{phox} and p67^{phox}, p67^{phox} alone, or Noxa1 alone or the absence of these cofactors. The siRNA-inhibited Nox3 activity, supported by p47^{phox} and p67^{phox}, was restored by transfection of wild-type Rac1. Finally, Nox3 activity supported by p47^{phox} or Noxo1 alone was also enhanced by Rac1(Q61L), both in HEK293 and in CHO-K1 cells, and moderately inhibited (30 to 40%) by RNAi in the HEK293 cell model (both vector-based and synthetic Rac1-specific siRNAs; data not shown). Because Noxa1 inhibits Nox3 activity supported by Noxo1, we did not obtain evidence of Rac1 involvement in Nox3 regulation when all three proteins were coexpressed, consistent with the findings of Ueno et al. (55). Indeed, any mutation that disrupts Noxa1 interactions with other oxidase components leads to enhanced Nox3 activity, which we attribute to the relief of its inhibitory effects on Nox3 and Noxo1 [i.e., Noxa1(R103E), Noxa1(W436R), Noxa1(R103E, W436R) (data not shown)]. We showed moderate inhibition of Nox3 activity supported by p47^{phox} and p67^{phox}(R102E) (without transfection of p22^{phox}) in HEK293 and CHO-K1 cells, while Ueno et al. showed only slight inhibitory effects of p67^{phox}(R102E) in Nox3 activity supported by overexpression of p22^{phox}, p47^{phox}, and p67^{phox} in CHO-K1 cells. Ueno et al. (55) argued against involvement of Rac1 in the Nox3 system by showing that the dominant-negative [Rac1(T17N)] and constitutively active [Rac1(Q61L)] forms of Rac1 did not affect this enzyme reconstituted in CHO-K1 or HeLa cells, while these proteins did affect the Nox2-based systems. We were unable to enhance the activity of any Nox3 system in HEK293 cells that approached maximum levels by overexpression of constitutively active Rac1(Q61L) [i.e., Nox3 plus Noxa1(pp), Nox3 plus p67^{phox}, and Nox3 plus p67^{phox}(pp) (data not shown)]. However, Rac1(Q61L) does enhance the activity of Nox3 alone or Nox3 supported by Noxa1 alone in both HEK293 and CHO-K1 cells. Cheng et al. reported that the activity of Nox2 requires constitutively active Rac1(G12V) in HEK293H cells; on the other hand, Nox3 does not (15). Previous reports and the present study show that Nox1 does not require transfected active Rac1 to reconstitute the fully active system (14, 21, 54). Thus, it appears that Nox3 has a lower demand for Rac1, in comparison with Nox1 or Nox2, and that HEK293 cells have sufficient endogenous active Rac1 to support Nox3, even in the presence of the overexpressed mutants.

It is interesting that the phagocytic oxidase paradigm for the subcellular localization of phox regulators also applies to the Nox regulators, whereby Noxo1 targets Noxa1 to the plasma membrane, analogous to p47^{phox} linking p67^{phox} to the membrane. Our observations on the independent colocalization of Rac1 and Noxa1 or p67^{phox} on membrane ruffles, when expressed in the absence of organizer proteins, may indicate that Rac1 acts together with these activators on other nonoxidase effectors at these subcellular sites. As noted above, the true physiological partners of Nox3 remain unclear, since differences across species were reported in the oxidase regulators detected in the inner ear; all four regulators have been detected in the mouse inner ear, where this oxidase has been implicated, while only p47^{phox} and Noxa1 expression was detected in the rat inner ear (8, 13). Our clear demonstration of Rac1 regulation in concert with either "activator" component indicates that Nox3 has retained this functional property shared by Nox1 and Nox2. Our observations on Rac1 involvement in Nox3 in the absence of transfected "activators" raise the interesting possibilities that some other undefined Rac-dependent "activator" supports Nox3 or that Rac1 can interact directly with Nox3. Further studies are needed to appreciate the significance of Rac1-based regulation of Nox3 in a physiological context.

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