Nox1-dependent Reactive Oxygen Generation Is Regulated by Rac1*

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Rac1 has been implicated in the generation of reactive oxygen species (ROS) in several cell types, but the enzymatic origin of the ROS has not been proven. The present studies demonstrate that Nox1, a homolog of the phagocyte NADPH-oxidase component gp91phox, is activated by Rac1. When Nox1 is co-expressed along with its regulatory subunits NOXO1 and NOXA1, significant ROS generation is seen. Herein, co-expression of constitutively active Rac1(G12V), but not wild-type Rac1, resulted in marked further stimulation of activity. Decreased Rac1 expression using small interfering RNA reduced Nox1-dependent ROS. CDC42(G12V) failed to increase activity, and small interfering RNA directed against CDC42 failed to decrease activity, pointing to specificity for Rac. TPR domain mutants of NOXA1 that interfere with Rac1 binding were ineffective in supporting Nox1-dependent ROS generation. Immunoprecipitation experiments demonstrated a complex containing Rac1(G12V), NOXO1, NOXA1, and Nox1. CDC42(G12V) could not substitute for Rac1(G12V) in such a complex. Nox1 formed a complex with Rac1(G12V) that was independent of NOXO1 and NOXO1, consistent with direct binding of Rac1(G12V) to Nox1. Rac1(G12V) interaction with NOXA1 was enhanced by Nox1 and NOXO1, suggesting cooperative binding. A model is presented comparing activation by regulatory subunits of Nox1 versus gp91phox (Nox2) in which Rac1 activation provides a major trigger that acutely activates Nox1-dependent ROS generation.

Rho family GTPases are implicated in innate immunity, regulation of cell shape and migration, and mitogenic regulation (1–3). Rac1 and Rac2 participate in the regulation of ROS generation in several cell types (4, 5), especially in the neutrophil, where Rac2 provides one of several “triggers” for activation of the phagocyte respiratory burst oxidase, a superoxide-generating NADPH-oxidase that participates in host defense against invading microbes. In addition to regulation of ROS production in phagocytes, there is growing evidence for Rac1 regulation of NADPH-oxidase activity (4, 5), especially in the neutrophil, where Rac2 provides one of several “triggers” for activation of the phagocyte respiratory burst oxidase, a superoxide-generating NADPH-oxidase that participates in host defense against invading microbes. In addition to regulation of ROS production in phagocytes, there is growing evidence for Rac1 regulation of ROS generation in several cell types. For example, Ras-transformed fibroblasts overproduce superoxide, and ROS generation is inhibited by a dominant negative mutant form of Rac1 (6); also, stimuli that increase Rac1-GTP in gastric epithelial cells increase ROS production (7). Muta-
require cell activation for assembly at the membrane. This is consistent with the structure of NOXO1, which lacks an autoinhibitory region (AIR) that is present in p47phox and is the target of regulatory phosphorylations. In naive phagocytes, the AIR binds internally to the tandem Src homology 3 region (hs-Src homology 3) of p47phox, blocking its interaction with p22phox. Phosphorylation of AIR upon phagocyte activation relieves this inhibition, permitting association and assembly to occur. Therefore, the absence of the AIR in NOXO1 probably accounts in part for the ability of NOXO1 to assemble with Nox1 in resting cells (23). In addition, the PX domain of NOXO1 binds to phospholipids that are present in naive cells, allowing localization of NOXO1 to the membrane (23). Whereas less is known about NOXA1, this protein has an activation domain that is highly homologous to that present in p67phox. NOXA1 also contains a TPR domain that associates with Rac1 in yeast two-hybrid and pull-down assays (26). However, whether the NOXA1-Rac1 complex is functional in Nox1-dependent ROS production has not been demonstrated.

Interestingly, in reconstitution studies using transfected cells (23, 24, 26), co-expression of Nox1, NOXO1, and NOXA1 results in the production of relatively high levels of reactive oxygen in several cell types without the need to co-express a small GTPase as Rac1. In contrast, when gp91phox is expressed in HEK293 cells along with its regulatory subunits, there is an absolute requirement for co-expression of activated Rac in order to observe reactive oxygen generation (27). The above results call into question whether a small GTPase is indeed necessary for NOX1-dependent ROS production. Therefore, the present studies were designed to investigate whether a small GTPase is indeed necessary for NOX1-dependent ROS production.

**Experimental Procedures**

Cells—HEK293 cells (Invitrogen) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Vectors Encoding Nox Enzymes and Regulatory Proteins—Cloning and subcloning of Nox1, Nox2 (gp91phox), NOXO1, NOXA1, p47phox, and p67phox were previously described (23, 27). (Myc)-Rac1(G12V), Myc-Rac1 wild-type, and (HA)3-CDC42(G12V), in pcDNA3.1 were from the University of Missouri-Rolla cDNA Resource Center (Rolla, MO). (HA)3-Rac1(G12V) was constructed by amplyifying HA and Rac1(G12V) separately and inserting these PCR products into pcDNA3.1. (HA)3_NOXO1 was constructed by inserting the NOXO1 PCR product into pCMV5-(HA)3, Myc-NOXO1 was constructed by inserting the PCR product of NOXO1 into the vector, pRK5(Myc). pRK5-Myc-PAK1 WT was kindly provided by Dr. Gary Bokoch (Scripps Research Institute). NOXA1(D68A) in pCMV-Sport6 was constructed by amplifying NOXA1 in pCMV-Sport6 using an SP6 primer and primer 1 (’5’-AAG TTG GCC ACT CCT CGC TGG AAG AAG CCA ACC GCC ATG CAG GTG GCC TTT GTC ACG GCT TG-3’). Primer 1 introduces an Mscl site (underlined) and changes (italics) codon 68 in NOXA1 from aspartic acid (D) to alanine (A). The PCR product was digested with Xhol and Mscl. The digested fragment was inserted into Xhol and Mscl sites of pCMV-Sport6/NOXA1 to replace the wild-type region. NOXA1(R103E) and NOXA1(D109A) in pCMV-Sport6 were made using an analogous strategy. NOXA1(D109A) also has a E100G mutation accidentally introduced by PCR.

**Measurement of Reactive Oxygen Species**—Reactive oxygen was measured using luminol chemiluminescence as previously described (27).

**Mammalian Two-hybrid Assay**—The pM cloning vector (Clontech) contains a GAL4 DNA binding domain, and the vector of pVP16 (Clontech) encodes an activation domain from Herpes simplex virus V16 protein. Both pM and pVP16 were modified by adding KpnI sites in the multiple cloning sites upstream of their BamHI sites. pG5CAT expresses a fusion protein consisting of five consensus GAL4 binding sites fused to chloramphenicol acetyltransferase (CAT). pM3-VP16 is a positive control vector that expresses a fusion of the GAL4-BD and the VP16-AD. Full-length NOX1 and its mutants, including NOXA1(D68A), NOXA1(R103E), and NOXA1(E100G/D109A), were subcloned into KpnI and BamHI sites of pM, whereas Rac1(G12V) and CDC42(G12V) were subcloned into KpnI and BamHI sites of pVP16. For detecting protein-protein interactions, HEK293H cells were co-transfected with 0.2 μg of pG5CAT along with 0.5 μg of pM (or its derivatives) plus 0.5 μg of pVP16 (or its derivatives) as indicated. Cells were harvested after 48 h and stored at −80 °C. CAT activity was assayed using the Fast CAT (deoxy)chloramphenicol acetyltransferase assay kit (Molecular Probes, Inc.). Briefly, frozen cells were resuspended in 100 µl of 0.25 M Tris-HCl, pH 7.4, and lysed with three freeze-thaw cycles and centrifuged at 12,000 rpm for 5 min at 4 °C. 60 µl of supernatant was mixed with 10 µl of FAST CAT substrate solution and preincubated at 37 °C for 5 min. Then 10 µl of freshly prepared 9 mM acetyl-CoA was added and incubated at 37 °C for 2–3 h. The reaction was stopped by adding 1 ml of ice-cold ethyl acetate. The liquid phases were separated by centrifugation at 12,000 rpm for 3 min. 900 µl of ethyl acetate containing the reaction product was dried under vacuum, and the residue was redissolved in 20 µl of ethyl acetate. A 5-µl aliquot was applied to a silica gel-60 thin layer chromatography plate (Merck), and separated using 85:15 (v/v) chloroform/methanol. The separated substrate and product were visualized under UV light and photographed using an AlphaimagerTM (Alpha Innotech Corp.).

**Immunoprecipitation**—HEK293H cells grown to ~50% confluence on 10-cm plates were transfected with 5 µg of each plasmid indicated (Fig. 5), using FuGene 6. After 48 h, the cells were harvested by washing twice with cold Hanks’ balanced salt solution. The cells were lysed in 600 µl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA) with protease inhibitor mixture (Sigma). MgCl₂ (60 mM) was included in the RIPA buffer for those experiments where Rac1 WT or endogenous Rac1 binding was examined. The lysate was centrifuged at 13,000 rpm for 15 min at 4 °C, and 1.5 mg protein of cleared lysate was used for each immunoprecipitation. The lysates were incubated overnight at 4 °C with primary antibodies with end-over-end rotation. The next morning, either 15 µl of protein G-Sepharose beads (1:1 slurry; Sigma) or 20 µl of streptavidin-agarose beads (Molecular Probes) was added to the mixtures and rotated for an additional 2.5 h at 4 °C. Beads were pelleted by centrifuging at 1,500 × g for 2 min and washed three times with cold RIPA buffer. The pellets were resuspended in 25 µl of RIPA buffer or RIPA buffer...
with 20 mM biotin. The immunocomplexes were analyzed by Western blotting and visualized by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce).

Antibodies—3 mg each of chicken anti-NOX01 and anti-NOX1 antibodies (23, 27) were separately biotinylated using the DBS-X Biotin Protein Labeling Kit (Molecular Probes) according to the manufacturer’s instructions and were used to immunoprecipitate untagged NOX01 and NOX1A, respectively. Anti-Nox1 E39.1 monoclonal antibody was previously described (23) and was the kind gift of Jackie Papkoff at DiaDexus (S. San Francisco, CA). Anti-green fluorescent protein polyclonal antibody was purchased from ANASPEC, Inc. (San Jose, CA). Anti-Myc and anti-HA monoclonal antibodies were from Cell Signaling Technologies, Inc. (Beverly, MA). Anti-Rac1 and anti-CDC42 antibodies were from Upstate Biotechnology, Inc. (Charlottesville, VA).

Rac Activation Assay—The endogenous GTP-associated form of Rac1 was detected using Rac/CDC42 Assay kit (Upstate Biotecnology), following the manufacturer’s protocol. Similar to the above immunoprecipitation, HEK293H cells grown to confluence on 10-cm plates were harvested by washing twice with Hanks’ balanced salt solution. The cells were lysed in 1 × MgCl₂ lysis buffer supplied by the manufacturer and supplemented with protease inhibitor mixture. The lysate was centrifuged at 13,000 rpm for 15 min at 4 °C. A total of four samples were prepared for each condition described in the legend to Fig. 1A. Two equal aliquots of the lysate were preloaded with either GTPγS or GDP as controls. A third equal aliquot was not preloaded. All three aliquots were then shaken with glutathione S-transferase-PAK1(67–150) bound to glutathione-agarose beads at 4 °C for 1 h. The fourth sample was composed of one-twenty-fifth of the volume of lysate that was used in the other three samples for the pull-down and was used directly for Western blotting. Beads were pelleted by centrifuging at 1,500 × g for 2 min and washed three times with cold MgCl₂ lysis buffer provided by the kit. The pellets were resuspended in 40 μl of SDS-PAGE loading buffer and subjected to Western blotting. Rac1-GTP was visualized using an anti-Rac1 monoclonal antibody supplied with the kit (Upstate Biotechnology).

Western Blot Analysis—Cells were lysed in RIPA buffer with protease inhibitor mixture (Sigma). Lysate (60 μg of protein) was resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane using a semidy electrophoretic transfer cell (Bio-Rad) at 15 V for 1 h. In some experiments, 40 μg of cell lysate was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using the Tank Transfer System (Bio-Rad). The proteins were detected using standard Western blotting and visualized by chemiluminescence as described above. Blots were stripped and reprobed as necessary.

RNA Interference of Rac1 and CDC42—Nonspecific randomized control siRNA and Rac1 siRNA were purchased from Upstate USA, Inc. (catalog no. M-003560), whereas siRNA of CDC42 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (catalog no. sc-29256). 200 pmol of control siRNA, Rac1 siRNA, and/or CDC42 siRNA were co-transfected into HEK293H cells in a 6-well plate with 0.5 μg each of Nox1, NOX01, and NOXA1 using X-tremeGENE siRNA transfection reagent (Roche Applied Science) according to the manufacturer’s instruction. After 72 h, the cells were harvested as described above. An aliquot of the cells was subject to luminol assay, and the remainder was used for Western blotting.

RESULTS

Stimulation of Nox1-dependent ROS Production by Rac1—We previously reported that co-transfection of Nox1, NOX01, and NOXA1 resulted in significant generation of ROS (23). In these earlier experiments, we failed to observe stimulation of Nox1-dependent ROS production by Rac1(G12V). Because these experiments used high concentrations of plasmids encoding Nox1, NOX01, and NOXA1, this may have artifically saturated Nox1 with its regulatory subunits, preventing further activation by Rac1. We therefore used relatively low concentrations of each plasmid in the present study. As shown in Fig. 1A, whereas significant ROS generation was still seen without co-expressed Rac1 and without phorbol 12-myristate, 13-acetate (PMA) stimulation (Fig. 1A, lane 2), activity was increased slightly by expression of wild-type Rac1 (lane 3) and was further increased by expression of Rac1(G12V) (lane 4).

To test whether there may have been endogenous Rac1-GTP present in these cells that might account for partial activation of Nox1 in the absence of Rac1(G12V), the content of endogenous Rac1GTP was evaluated using the Rac binding domain of PAK (PAK–(67–150)). This binding domain interacts exclusively with the GTP form of Rac1 and CDC42. As shown in the Western blot in Fig. 1B (top), Rac1 was seen in both the total cell lysate (lane 1) and in the PAK1 pull-down complex from lysates of untreated cells (lane 3). Because the pull-down complex was concentrated 25-fold compared with lysate and gave about 5% of the staining on the Western blot, compared with the band seen in the lysate, one can estimate that ~0.2% of endogenous Rac1 is in the GTP-bound form. This was not affected by co-expression of Nox1, NOXA1, and NOXO1 (Fig. 1B, second panel). Co-transfection of Myc-tagged wild-type Rac1 WT along with Nox1, NOXA1, and NOXO1 did not result in an increase in the level of activated endogenous Rac1, but some activated Myc-tagged Rac1 WT was seen (Fig. 1B, third panel from the top). Finally, when Myc-Rac1(G12V) was co-expressed along with Nox1, NOXA1, and NOXO1, the PAK1 pull-down revealed both Rac1(G12V) and an increased level of endogenous activated Rac1 (Fig. 1B, bottom). For unknown reasons, Rac1(T17N) failed to inhibit ROS generation when co-expressed with Nox1, NOXA1, and NOXA1, perhaps because this cell type may utilize a guanine nucleotide exchange factor that is insensitive to inhibition by Rac1(T17N).

The concentration dependence for Rac1(G12V) activation of Nox1 with and without PMA stimulation is shown in Fig. 1C. Without PMA, Nox1-dependent ROS generation was stimulated by Rac1(G12V) by ~3-fold at the highest concentrations tested (1,000 ng). Rac2, which is expressed exclusively in phagocytic cells (28), produced a similar -fold activation and dose dependence (data not shown). PMA caused up to 2-fold increase in basal activity in the absence of Rac1(G12V), and activity increased by a roughly constant amount throughout the Rac1(G12V) concentration range.

We previously showed that Nox2, when co-expressed with p47phox, p67phox, and Rac1(G12V), can be activated by PMA (27). As a control, we tested the dose dependence for activation of Nox2 by Rac1(G12V), as is shown in Fig. 1D. Nox2 activation absolutely required stimulation by PMA, and no basal activity was seen in the absence of Rac1(G12V). Thus, the quantity of endogenous Rac1-GTP in unstimulated cells is not sufficient to activate Nox2. The absolute requirement of PMA for Nox2, but not Nox1, activation is not surprising, since PMA-dependent phosphorylation of the AIR of p47phox and perhaps other components is well known (29, 30), whereas the AIR is absent in NOXO1. Interestingly, in a transgenic COS7 cell line stably expressing gp91phox, p47phox, p22phox, p40phox, and p67phox, stimulus-independent ROS production was induced by transfection of Rac1(G12V) (31). In these COSphox cells, transfection of Rac1(G12V) drove translocation of p47phox to the membrane, suggesting that Rac1 acts not only as a participant in the NADPH oxidase complex but also as a regulator of oxidase assembly.

We were not able to compare the protein expression levels of Nox1 versus Nox2 in Fig. 1D due to the lack of an antibody that recognizes the
same epitope on both proteins. Apart from the differential dependence on PMA, the most striking difference between Fig. 1, C and D, is that the curve for Nox2 activation by Rac1(G12V) shows typical saturation kinetics, whereas Nox1 does not. Differences in localization of NOXA1/NOXO1 versus p67phox/p47phox could be one reason for the difference in kinetics. Since p67phox/p47phox is cytosolic and translocates to the membrane during PMA stimulation, a limiting amount of translocated p67phox/p47phox could limit the amount of Rac1 required and account for the apparent saturation observed in Fig. 1D. NOXA1/NOXO1, on the other hand, is localized at the membrane, as we have previously shown, and this might account for the nonsaturable kinetics displayed in Fig. 1C. Alternatively, Rac1 might regulate Nox1 by mechanisms in addition to direct complex formation, such as affecting localization, cytoskeletal structures, etc.

Decreased Expression of Endogenous Rac1 Inhibits Activation of Nox1—Since we observed a measurable level of endogenous Rac1-GTP, we hypothesized that the basal Nox1 activity in the absence of co-transfected Rac1(G12V) was due to endogenous Rac1. To test this hypothesis, Rac1 siRNA, CDC42 siRNA, or a scrambled control siRNA was co-transfected along with Nox1, NOXO1, and NOXA1 (Fig. 2). The scrambled siRNA-treated cells in this experiment produced nearly the same ROS as the cells with no siRNA (not shown). Although it was not possible to achieve complete elimination of Rac1 expression, co-transfection of Rac1 siRNA inhibited Nox1-dependent ROS generation by about 50% compared with control siRNA, and this corresponded to a roughly 50% reduction in the level of endogenous Rac1 (Fig. 2). CDC42 siRNA did not inhibit ROS generation; in fact, it slightly increased ROS generation when transfected alone (lane 5), consistent with the slightly higher ROS generation in lane 4 versus lane 3, where the siRNA for CDC42 was co-transfected with or without the siRNA for Rac1, respectively. Western blotting confirmed the specific knockdown of endogenous Rac1 or CDC42 without affecting the expression of NOXA1 or NOXO1 proteins (Fig. 2, bottom).
NOXA1. These residues were mutated to alanine residues in codons 68 and 109 (the Arg109 mutation also contained an additional PCR-introduced mutation at codon 100 converting glutamate to glycine) and to glutamate at codon 103. The NOXA1 mutants were tested for their ability to reconstitute ROS generation when co-expressed with Nox1 and NOXO1. As shown in Fig. 3, each of these NOXA1 mutants showed markedly decreased or absent ability to support Nox1-dependent ROS generation. The Western blot in Fig. 3 shows that the NOXA1 mutants were expressed in amounts similar to or higher than wild-type NOXA1.

**Rac1 Binding to NOXA1**—Fig. 4A uses the mammalian two-hybrid system to detect binding between Rac1 and full-length wild-type NOXA1 (lane 5, upper spot). Mutation of NOXA1 within the TPR domain at Asp68 (lane 6), Arg109 (lane 7), or Asp109/Glu100 (lane 8) eliminates detectable binding of NOXA1 to Rac1. CDC42 has a similar structure and is 69% identical to Rac1. CDC42 was cotransfected into HEK293H with NOX1, NOXO1, and NOXA1 as described under “Experimental Procedures.” After 72 h, ROS was measured by luminol chemiluminescence (top) as in Fig. 1. The bottom panel shows a representative Western blot. Lane 1, nontransfected cells; lane 2, Nox1/NOXO1/NOXA1/Nox1 siRNA; lane 3, Nox1/NOXO1/NOXA1/Rac1 siRNA; lane 4, Nox1/NOXO1/NOXA1/Rac1 siRNA/CDC42 siRNA; lane 5, Nox1/NOXO1/NOXA1/CDC42 siRNA. The expression of untagged Nox1 could not be demonstrated at the low expression level used in this experiment. Data are representative of three independent experiments, and error bars show S.E. (n = 3). RLU, relative luminescence units.

**Association of Rac1 in a Multimeric Complex with Nox1, NOXA1, and NOXO1**—To investigate further the interactions between Nox1, NOXO1, NOXA1, and Rac1, immunoprecipitation studies were conducted in transiently transfected HEK293H cells. We first examined the complexes formed between NOXA1, NOXO1, and Rac1(G12V) in cells co-transfected with Nox1, NOXO1, and NOXA1, either with or without Rac1(G12V). As shown in Fig. 5A, the protein complex immunoprecipitated using an anti-Myc antibody directed against (Myc)2-Rac1(G12V) contained NOXA1 as well as a small amount of NOXO1, indicating an association among these three proteins. Confirming this result, immunoprecipitation of NOXA1 yielded a complex in which both Rac1(G12V) and NOXO1 were detected, and immunoprecipitation of NOXO1 showed an association with NOXA1 and Rac1(G12V).

The interactions among these regulatory proteins and Nox1 were explored using epitope-tagged versions of the regulatory proteins as well as an N-terminal fusion protein between EGFP and Nox1. We previously showed that EGFP-Nox1 is highly active in HEK293 cells (33), and we have found that it improves migration on SDS gels, facilitating detection of Nox1 on Western blots.4 In addition, epitope-tagged forms of the regulatory proteins were active in supporting ROS production by EGFP-Nox1 (data not shown). Fig. 5B shows the interaction of Nox1 and NOXA1. By immunoprecipitating either component, NOXO1 and Nox1 co-precipitated both in the presence and absence of Rac1(G12V) (lanes 2 and 5). As expected, the interaction between these two proteins was not observed when either Nox1 or NOXA1 was omitted from the transfection protocol (lanes 3 and 4).

**FIGURE 2.** Decreased expression of Rac1 inhibits activation of Nox1. siRNA for Rac1 or for CDC42 was cotransfected with Nox1, NOXO1, and NOXA1 as described under “Experimental Procedures.” After 72 h, ROS was measured by luminol chemiluminescence (top) as in Fig. 1. The bottom panel shows a representative Western blot. Lane 1, nontransfected cells; lane 2, Nox1/NOXO1/NOXA1/Nox1 siRNA; lane 3, Nox1/NOXO1/NOXA1/Rac1 siRNA; lane 4, Nox1/NOXO1/NOXA1/Rac1 siRNA/CDC42 siRNA; lane 5, Nox1/NOXO1/NOXA1/CDC42 siRNA. The expression of untagged Nox1 could not be demonstrated at the low expression level used in this experiment. Data are representative of three independent experiments, and error bars show S.E. (n = 3). RLU, relative luminescence units.

**FIGURE 3.** Predicted Rac-binding mutations in the TPR domain of NOXA1 inhibit Nox1-dependent ROS generation. Nox1 and NOXO1 were co-transfected along with empty vector (−) or vector encoding wild-type NOXA1 or NOXA1 mutated at the indicated positions in its TPR domain. ROS generation was measured as in Fig. 1. Open bars, in the absence of PMA; filled bars, after the addition of 200 nm PMA. Data are representative of three independent experiments, and error bars show the S.E. (n = 3). In the lower panels, Western blots were used to visualize NOXA1 and its point mutants as well as tubulin as a loading control. RLU, relative luminescence units.

4 T. Kawahara, D. Ritsick, G. Cheng, and J. D. Lambeth, unpublished observations.
amount of EGFP-Nox1 was detected when (Myc)-Rac1 WT was used in place of (Myc)2-Rac1(G12V) (lane 7), consistent with the activation of a fraction of the overexpressed Rac1 WT (Fig. 1B). We could not detect an interaction between endogenous Rac1 and EGFP-Nox1 (lane 6) due to the small amount of endogenous Rac 1-GTP that is in these cells (Fig. 1B).

The second panel from the top in Fig. 5C shows the interaction between Rac1 and NOXA1. Interestingly, (HA)3-NOXA1 co-precipitated with (Myc)2-Rac1(G12V) only when both Nox1 and NOXO1 were present (compare lanes 2, 3, and 5). A small amount of NOXA1 co-precipitated with overexpressed Rac1 WT (lane 7), but not with endogenous Rac1 (lane 6). Our observation that NOXA1 and Rac1 do not bind to each other in the absence of Nox1 and NOXO1 may indicate that the interaction between NOXA1 and Rac1 is cooperative with respect to Nox1 and NOXO1. Although the direct interaction between NOXA1 and Rac1 in the absence of Nox1 and NOXO1 was not seen in immunoprecipitation studies, this interaction was readily observed using the mammalian two-hybrid method above and by yeast two-hybrid and glutathione S-transferase pull-down assays (26).

Fig. 5D shows that CDC42(G12V) does not form a complex with the complex of Nox1, NOXO1, and NOXO1. An anti-HA antibody was used to immunoprecipitate either (HA)3-CDC42(G12V) (lanes 2 and 3) or (HA)2-Rac1(G12V) (lane 4). In the presence of NOXA1 and NOXO1, EGFP-Nox1 was detected only in the immunoprecipitate containing Rac1(G12V) and not that containing CDC42(G12V) (lane 4 versus lane 2), in agreement with the mammalian two-hybrid assay (Fig. 4). To demonstrate that CDC42(G12V) was in the active conformation, Myc-PAK1 was co-transfected with (HA)3-CDC42(G12V) in the same experiment. The third panel shows that Myc-PAK1 co-precipitated with (HA)3-CDC42(G12V).

Fig. 5E investigates interactions of NOXO1 with Nox1 and NOXA1. An anti-Myc antibody was used to immunoprecipitate Myc-NOXO1. Nox1 co-immunoprecipitated with NOXO1 when Nox1 and Rac1(G12V) were present (Fig. 5E, top, lane 2). In the absence of NOXO1 (top panel, lane 4) or in the absence of Rac1(G12V) (lane 6), Nox1 still co-immunoprecipitated with NOXO1. This indicates that NOXO1 interacts with Nox1 independently of NOXA1 and Rac1(G12V). Since p22GFF is expressed constitutively in these cells, this interaction is likely to be mediated in part by this protein. In addition, it was recently reported (36) that NOXO1 binds to the C terminus of Nox1 in the vicinity of the NADPH binding site. Panel 2 of Fig. 5E shows that NOXO1 immunoprecipitated with NOXO1 whether or not Nox1 or Rac1G12V were present (compare lanes 2, 3, and 6).

Fig. 5F summarizes the results from our immunoprecipitation experiments. The solid line between two proteins indicates an independent interaction, whereas a dotted line indicates a cooperative interaction as follows: 1) Nox1 and NOXA1 interact in the absence of Rac1 (Fig. 5B); 2) Rac1 interacts with Nox1 in the absence of NOXO1 (Fig. 5C, top); 3) Rac1(G12V) interacts with NOXA1 only when Nox1 and NOXO1 are in the complex. (Fig. 5C, second panel); 4) NOXO1 interacts with Nox1 in the absence of NOXA1 or in the absence of Rac1(G12V) (Fig. 5E, top); 5) NOXO1 interacts with NOXO1 in the absence of Nox1 or in the absence of Rac1 (Fig. 5E, second panel).

**DISCUSSION**

The present studies provide evidence that activated Rac1 forms a multimeric complex with Nox1, NOXO1, and NOXO1 and that Rac1 regulates Nox1-dependent ROS production in transfected HEK293 cells. The failure to observe Rac regulation in earlier studies was probably due to high levels of Nox components and to the presence of Rac1-GTP under cell culture conditions. With the lower expression of Nox components in this study, constitutively active Rac1(G12V) was able to stimulate ROS generation significantly. Although basal activity was seen when Nox1 was co-transfected with NOXO1 and NOXO1 in the absence of Rac1(G12V), this activity also is likely to be due to endogenous Rac1 that is expressed constitutively in these cells and that is about 0.2% in the GTP-bound, activated form. Consistent with this interpretation, cellular expression of PAK-(67–150), which binds selectively to Rac1-GTP and CDC42-GTP, inhibited Nox1-dependent ROS production. These data are consistent with Rac1-GTP being the form of Rac that participates in activating Nox1. However, the effects were complex, since the PAK binding domain also decreased expression of Nox components somewhat, although to a lesser extent than the effects on activity.

Whereas small GTPases also act on a variety of targets, such as cytoskeleton, PAK, JNK, etc., and hence might act on ROS generation indirectly, the present studies provide evidence that Rac1 activates the Nox1 system as a member of a multimeric Nox1 complex. The mammalian two-hybrid system demonstrates direct binding of Rac1 (but not
FIGURE 5. Co-immunoprecipitation of Nox1, NOXO1, NOXA1, and Rac1. HEK293H cells were co-transfected with plasmids encoding the indicated proteins. The antibodies used for immunoprecipitation (IP) and Western blotting (WB) of the co-precipitated complexes and of the lysates are indicated in the figure. All experiments shown are representative of at least three independent experiments. A, cells were co-transfected with Nox1, NOXA1, and NOXO1, either with or without (Myc)2-Rac1(G12V). Immunoprecipitation was carried out.

|                | IP: Anti-myc (Rac1) | WB: Anti-NOXO1 | WB: Anti-NOXA1 | WB: Anti-Nox1 (E39) |
|----------------|---------------------|----------------|----------------|---------------------|
| Nox1           | +                   | +              | +              | +                   |
| NOXO1          | +                   | +              | +              | +                   |
| NOXA1          | +                   | +              | +              | +                   |
| myc(Rac1)      | +                   |                | +              | +                   |

B

|                | IP: Anti-EGFP (Nox1) | WB: Anti-NOXO1 | WB: Anti-NOXA1 | WB: Anti-Nox1 (E39) |
|----------------|----------------------|----------------|----------------|---------------------|
| Nox1           |                      | +              | +              | +                   |
| NOXO1          |                      |                | +              | +                   |
| NOXA1          |                      |                | +              | +                   |
| myc(Rac1)      |                      |                | +              | +                   |

C

|                | IP: Anti-myc (Rac1) | WB: Anti-NOXO1 | WB: Anti-NOXA1 | WB: Anti-Nox1 (E39) |
|----------------|---------------------|----------------|----------------|---------------------|
| Nox1           | +                   | +              | +              | +                   |
| NOXO1          | +                   | +              | +              | +                   |
| NOXA1          | +                   | +              | +              | +                   |
| myc(Rac1)      | +                   |                | +              | +                   |

D

|                | IP: Anti-HA (CDC42) | WB: Anti-NOXO1 | WB: Anti-NOXA1 | WB: Anti-Nox1 (E39) |
|----------------|---------------------|----------------|----------------|---------------------|
| Nox1           |                      | +              | +              | +                   |
| NOXO1          |                      |                | +              | +                   |
| NOXA1          |                      |                | +              | +                   |
| myc(Rac1)      |                      |                | +              | +                   |

E

|                | IP: Anti-myc (NOXO1) | WB: Anti-NOXO1 | WB: Anti-NOXA1 | WB: Anti-Nox1 (E39) |
|----------------|----------------------|----------------|----------------|---------------------|
| Nox1           |                      | +              | +              | +                   |
| NOXO1          |                      |                | +              | +                   |
| NOXA1          |                      |                | +              | +                   |
| myc(Rac1)      |                      |                | +              | +                   |

F

[Diagram showing interactions between Nox1, NOXO1, and Rac1]
CDC42) to the NOX1 via the TPR region, since mutations in this region disrupted binding. This is in agreement with an earlier study that showed direct binding of Rac1 to NOX1 using the yeast two-hybrid method and by a glutathione S-transferase fusion protein pull-down method (26). However, the present immunoprecipitation studies demonstrated NOX1-Rac1 interactions only when Nox1 and NOXO1 were also both present, and no interaction was seen when either of these proteins was omitted. This indicates that the interaction between NOX1 and Rac1 is strongest when both Nox1 and NOXO1 are also present. Such a result is consistent with cooperative binding due to multisite interactions among protein components, since both Rac1 and NOX1 bind to two or more other proteins.

Based on the mutational analysis, Rac1-GTP binds to the TPR region of NOX1 in a manner analogous to Rac-GTP binding to p67phox. In the latter system, Rac and p67phox both provide binding sites for p67phox, facilitating its regulation of the activity of the gp91phox subunit by its activation domain. Rac provides a dual role, both tethering p67phox to the flavocytochrome (34, 35) and inducing an active conformation of p67phox (37). Because NOX1 also contains a highly homologous activation domain that is essential for regulating Nox1 activity (27), Rac1 probably plays an analogous role in regulating Nox1 activity via binding and orienting NOX1. Exogenous Rac1(G12V) enhanced Nox1 activity up to 3-fold above basal levels, and reduction of endogenous Rac1 expression by 50% using siRNA reduced Nox1 activity by ~50%. We cannot exclude the possibility that Nox1 can function in the absence of Rac1, since endogenous Rac1 was not completely eliminated by siRNA treatment. However, the correlation of residual Nox1 activity with the residual Rac1 level (both about 50%) suggests that if Rac1-independent activity exists, it is very low and probably negligible. Future studies using isolated components or using Rac1 knock-out cell lines will be able to address this question directly.

The present studies in combination with earlier studies suggest that activation of Rac1 by GTP loading may function as a major trigger for activating the Nox1 system, as shown in Fig. 6 (right). Whereas both the Nox1 system and the gp91phox system depend upon Rac activation, the “kinetics” of activation with respect to Rac1 appear to be quite different. In the gp91phox system, at least three downstream triggering events induce assembly and activation. These include guanine nucleotide exchange on Rac, phosphorylation of p47phox (and probably other components), and lipid phosphorylation to form 3′-phosphorylated phosphatidylinositol lipids to which the PX domain of p47phox binds. As shown previously, in HEK293 cells transfected with NOXO1, NOXA1, and Nox1, all of these subunits co-localize at the membrane and do not require cell stimulation for assembly. Whereas this might differ in other cell types, the structure and binding properties of NOX1 support the interpretation that the system is “designed” to bypass some of the activation triggers that are present in phagocytes. Specifically, NOX1 lacks the autoinhibitory region containing regulatory phosphorylation sites, and its PDZ domain binds to phosphatidylinositol-4-monophosphate, which is present in membranes in unstimulated cells. This structure probably accounts for the assembly of NOX1 with Nox1 at membranes. Thus, the Nox1 system in unstimulated cells is partially assembled, and GTP loading of Rac may be sufficient to complete the assembly process and induce activation of the enzyme (Fig. 6, right). The more complex regulation in phagocytes may represent a “fail-safe” system wherein multiple triggers must be simultaneously activated in order to “launch” the very robust and potentially tissue-damaging barrage of ROS. Since ROS levels generated by endogenous Nox1 are typically far lower than those produced by activated phagocytes, accidental activation of Nox1 is less likely to be catastrophic, and the mechanism of Nox1 regulation may be less complex. Although Rac1 activation is a major regulator of Nox1, we cannot rule out the contribution of other signaling events, such as phosphorylation.

Thus, Rac-regulated ROS generation may occur through at least two Nox proteins, Nox1 and gp91phox. However, not all Nox proteins depend on Rac for their activity. Our unpublished data do not support the possibility that Rac1 enhances Nox3-generated ROS production when HEK293 cells are cotransfected with Nox3 and NOXO1. Nox4 is activated by insulin in fat cells (38) and by angiotensin II (39) in mesangial cells. However, when expressed in HEK293 and other cells, Nox4 is constitutively active and does not show a requirement for NOXO1, NOXA1, or Rac1 (40). Nox5 is regulated by calcium, and reports of the effects of regulatory subunits or small GTPases are scarce. It was recently reported, however, that ROS generation by Nox5 in circulating malignant B cells was not affected by Rac1 inactivation by toxin B (41). Very little is known about the activation of Duox, which is also regulated by calcium. In dog thymocytes, it appears that Rac activation is not necessary for H2O2 production (42). Therefore, further investigations are needed to determine whether Nox1 and gp91phox are the major Nox proteins.
targets for Rac-stimulated ROS generation or whether other Nox enzymes are also regulated in a similar manner.

Addendum—While this paper was undergoing final revisions, a manuscript appeared in online form showing Rac1 regulation of Nox1 activity. In addition, Nox3-dependent ROS generation was regulated by Rac1 when NOXA1 was used as an activator but not when NOXO1 alone was the activating protein (23).

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