Direct Involvement of the Small GTPase Rac in Activation of the Superoxide-producing NADPH Oxidase Nox1*

Kei Miyano†‡, Noriko Ueno§, Ryu Takeya∥, and Hideki Sumimoto¶†

From the †‡Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, and §∥CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Received for publication, December 22, 2005, and in revised form, June 5, 2006 Published, JBC Papers in Press, June 8, 2006, DOI 10.1074/jbc.M513665200

Activiation of the non-phagocytic superoxide-producing NADPH oxidase Nox1, complexed with p22-phox at the membrane, requires its regulatory soluble proteins Noxo1 and Noxa1. However, the role of the small GTPase Rac remained to be clarified. Here we show that Rac directly participates in Nox1 activation via interacting with Noxa1. Electropermeabilized HeLa cells, ectopically expressing Nox1, Noxo1, and Noxa1, produce superoxide in a GTP-dependent manner, which is abrogated by expression of a mutant Noxa1(R103E), defective in Rac binding. Superoxide production in Nox1-expressing HeLa and Caco-2 cells is decreased by mutant Noxa1(R103E), defective in Rac binding. Superoxide production in Nox1-expressing HeLa and Caco-2 cells is decreased by mutation of a mutant Rac1 with the A27K substitution, deficient in binding to Noxa1. We also demonstrate that Nox1 activation requires membrane recruitment of Noxa1, which is normally mediated via Noxa1 binding to Noxo1, a protein tethered to the Nox1 partner p22-phox; the Noxa1-Noxo1 and Noxo1-p22-phox interactions are both essential for Nox1 activity. Rac likely facilitates the membrane localization of Noxa1: although Noxa1(W436R), defective in Noxo1 binding, neither associates with the membrane nor activates Nox1, the effects of the W436R substitution are restored by expression of Rac1(Q61L). The Rac-Noxa1 interaction also serves at a step different from the Noxa1 localization, because the binding-defective Noxa1(R103E), albeit targeted to the membrane, does not support superoxide production by Nox1. Furthermore, a mutant Noxa1 carrying the substitution of Ala for Val-205 in the activation domain, which is expected to undergo a conformational change upon Rac binding, fully localizes to the membrane but fails to activate Nox1.

Although reactive oxygen species (ROS) were previously considered to be by-products in aerobic metabolism, it has recently been accepted that ROS are also produced as true products by specialized enzymes, thereby participating in a variety of biological processes including host defense, hormone biosynthesis, oxygen sensing, and signal transduction (1–5). Enzymes dedicated to ROS production include members of the NAD(P)H oxidase (Nox) family, which are membrane-spanning flavocytochromes that reduce molecular oxygen to superoxide with electron derived from NAD(P)H (1–5).

The best characterized member of the family is gp91-phox, also termed Nox2, that functions as the catalytic subunit of the phagocyte NADPH oxidase (phox) (6–9). This oxidase plays a crucial role in host defense, which is evident from recurrent and life-threatening infections that occur in patients with chronic granulomatous disease, whose phagocytes genetically lack the superoxide producing activity (6–9). gp91-phox, stably complexed with the membrane-integrated protein p22-phox, is dormant in resting cells, but becomes activated during phagocytosis to produce superoxide, a precursor of microbicidal ROS.

Activation of gp91-phox/Nox2 requires the small GTPase Rac and the two specific adaptor proteins p47-phox and p67-phox, each containing two SH3 domains. These proteins, albeit existing in the cytoplasm of resting phagocytes, are targeted upon cell stimulation to the membrane to interact with the gp91-phox/p22-phox complex, which allows gp91-phox to transport electrons for superoxide production (10–16). The stimulus-induced membrane targeting of p47-phox and subsequent activation of gp91-phox require the interaction between p47-phox and p22-phox, which is mediated by binding of the p47-phox SH3 domains to the p22-phox C-terminal proline-rich region (PRR) (16–19); the substitution of Gln for Pro-156 in the PRR, a mutation found in a patient with chronic granulomatous disease, leads to a defective binding to p22-phox (16, 17). Upon cell stimulation, p67-phox translocates to the membrane via its association with p47-phox (21–25), which is crucial for oxidase activation (26). At the membrane, p67-phox directly interacts with GTP-bound Rac. This small GTPase is recruited to the membrane independently of p47-phox or p67-phox, and binds to the p67-phox N-terminal region of about 200 amino acid residues, containing four tetra tricopeptide repeat (TPR) motifs (27, 28). A mutant p67-phox carrying the substitution of Gln for Arg-102 in the third TPR neither interacts with Rac nor supports superoxide production by gp91-phox (27). Thus the Rac-p67-phox interaction plays an essential role in activation of gp91-phox (27–31). In addition, deletion of a region C-terminal to the Rac-binding domain in p67-phox (amino acid residues 200–212), so-called an activation domain, or the V204A substitution results in an impaired activation of gp91-phox (32, 33). Taken together,
Mechanism for Rac-mediated Regulation of Nox1

FIGURE 1. Domain structures of Nox1 and Noxa1. Nox1 harbors a PX domain, two SH3 domains, and a PRR. The SH3 domains bind to the PRR of p22\textsubscript{phox}. Noxa1 comprises a domain containing 4 units of tetratricopeptide repeat (TPR) motifs, an activation domain (AD), and an SH3 domain. Noxa1 interacts with GTP-bound Rac via the N-terminal TPR domain, whereas Noxa1 associates with Noxo1 via a tail-to-tail interaction of the Noxa1 SH3 domain with the Noxo1 PRR. T1 through T4 denote the first to fourth TPR motifs. Amino acid residues mentioned in the text are indicated by arrowheads.

Rac binding to the p67\textsubscript{phox} TPR domain seems to induce a conformational change of the activation domain in p67\textsubscript{phox}, thereby activating gp91\textsubscript{phox} (14, 33–35).

Nox1, the first identified gp91\textsubscript{phox} homologue in mammals, is abundantly expressed in colon epithelial cells and vascular smooth muscle cells (36, 37). Recent studies have shown that Nox1 participates in host defense at the colon (38, 39) and angiotensin II-mediated hypertension (40, 41). This non-phagocytic oxidase forms a heterodimer with p22\textsubscript{phox} (42–45), and can be activated by p47\textsubscript{phox} and p67\textsubscript{phox} but to a much lesser extent than gp91\textsubscript{phox}/Nox2 (42, 46, 47). Activation of Nox1 requires the regulatory proteins Noxo1 (Nox organizer 1) and Noxa1 (Nox activator 1), novel respective homologues of p47\textsubscript{phox} and p67\textsubscript{phox}, but not cell stimulants (42, 46–48). Nox1 is known to bind to p22\textsubscript{phox} via its tandem SH3 domains, whereas Noxa1 interacts with Noxo1 by binding of the Noxa1 SH3 domain to the Nox1 SH3 domain (42, 46–48). The roles of these interactions in Nox1 activation, however, have remained to be elucidated.

Rac is considered to be involved in ROS production also in non-phagocytic cells (1, 4, 49). This small GTPase, however, does not seem to be directly involved in activation of non-phagocytic oxidases such as Nox3 (50), Nox4 (51), and Duox, a distinctly related member of the Nox family (52). On the other hand, it has been reported that blockade of molecules acting upstream of Rac activation decreases superoxide production in Nox1-expressing cells, suggesting the involvement of Rac (53, 54). However, it has remained unknown how Rac functions in Nox1 activation.

We have previously demonstrated that GTP-loaded Rac directly interacts with the N-terminal TPR domain of Nox1 as well as that of p67\textsubscript{phox}; the interaction is abolished by substitution of Glu for Arg-103 (Ref. 42; see Fig. 1). In the present study, we show that electropermeabilized HeLa cells, which ectopically express Nox1, Noxo1, and Noxa1, produce superoxide in a GTP-dependent manner. The production is decreased when a mutant Noxa1(R103E) is expressed instead of the wild-type one, indicative of the role for Rac. Consistent with this, Nox1-dependent superoxide production by intact HeLa cells is decreased by depletion or sequestration of Rac, but enhanced by expression of the constitutively active Rac1(Q61L). We also demonstrate that Nox1 activation requires membrane recruitment of Nox1, which is normally mediated via Noxa1 binding to Nox1, a protein tethered to the Nox1 partner p22\textsubscript{phox}; the Noxa1-Nox1 and Nox1-p22\textsubscript{phox} interactions are both required for the superoxide producing activity of Nox1. Rac functions in Nox1 activation via its interaction with Noxa1: this GTPase likely acts by inducing a conformational change of the activation domain of Nox1; it also facilitates membrane localization of Nox1 in the absence of sufficient interaction between Nox1 and Noxa1.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The human cDNAs encoding Nox1, gp91\textsubscript{phox}/Nox2, p22\textsubscript{phox}, Nox1, Noxo1, and Noxa1 were ligated to the mammalian expression vector pcDNA3.0 (Invitrogen). The cDNA encoding Rac1, p22\textsubscript{phox}, Nox1, p47\textsubscript{phox}, and p67\textsubscript{phox} ligated to the mammalian expression vector pEF-BOS (57): Rac1 was constructed for expression as a myc-tagged protein; p22\textsubscript{phox} as a protein without a tag; Nox1 and p47\textsubscript{phox} as an HA-tagged protein; and Noxa1 and p67\textsubscript{phox} as a myc-tagged protein. Transfection of HeLa cells with the cDNAs was performed using Lipofectamine (Invitrogen), whereas FuGENE 6 Transfection Reagent (Roche Diagnostics) was used for transfection of CHO cells (58). When indicated, Nox1 was expressed as a FLAG-tagged protein using the vector pEF-BOS. Transfection of the human colon cancer Caco-2 cells with cDNAs for Nox1 and Noxa1 was performed with Cell Line Nucleofector™ Kit T (Amaxis) using the Nucleofector™ apparatus (Amaxis), according to the manufacturer’s instruction.

Estimation of Oxidase Proteins Expressed in HeLa, Caco-2, and CHO Cells—Total cell lysates of HeLa, Caco-2, and CHO cells were used for estimation of expression of Nox1, Noxo1, and Noxa1. The lysates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with an anti-HA monoclonal antibody (Covance Research Products), an anti-myc monoclonal antibody (Roche Diagnostics), an anti-FLAG monoclonal antibody (Sigma), anti-p22\textsubscript{phox} polyclonal antibodies (Santa Cruz Biotechnology), an anti-Rac monoclonal antibody (BD Bio-
Mechanism for Rac-mediated Regulation of Nox1

Superoxide Production by Electropermeabilized HeLa Cells Expressing Nox1, Noxo1, and Noxa1—The transfected HeLa cells were cultured for 30 h, and harvested by incubation with trypsin/EDTA for 1 min at 37 °C. Cell permeabilization was performed by the method of Grinstein and Furuya (59) with minor modifications. Briefly, the cells were suspended in an electroporation buffer (140 mM KCl, 1.0 mM MgCl₂, 0.2 mM CaCl₂, 1.0 mM EDTA, 1.0 mM NADPH, and 10 mM Hepes, pH 7.4) containing 0.5 mM GTP or 2.5 mM GDP, and transferred to a Bio-Rad Gene Pulser and permeabilized with a discharge of 5 kV/cm from a 25-μF capacitor. After being washed with the electroporation buffer, the cells were tested for estimation of superoxide producing activity. The activity was determined by superoxide dismutase-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics), as previously described (42, 50). The chemiluminescence was assayed at 37 °C using a luminometer (Auto Lumat LB953; EG&G Berthold).

Superoxide Production by Cells Expressing Nox1 or gp91phox—The transfected cells were cultured for 30 h, and harvested by incubation with trypsin/EDTA for 1 min at 37 °C. After being washed with Heps-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl₂, 0.5 mM CaCl₂, and 17 mM Hepes, pH 7.4), the cells were suspended at the concentration of 8 × 10⁵ cells/ml (HeLa and CHO cells) or 2 × 10⁶ cells/ml (Caco-2 cells), and preincubated for 5 min at 37 °C. The superoxide producing activity was determined by superoxide dismutase-inhibitable chemiluminescence with DIOGENES. The chemiluminescence was measured for 10 min at 37 °C using the luminometer. For estimation of superoxide production by gp91phox/Nox2, the transfected cells were stimulated with phorbol 12-myristate 13-acetate (Research Biochemicals International) at 200 ng/ml (50, 58).

RNA Interference (RNAi) for Knockdown of Rac1 and Cdc42—As double strand small interfering RNA (siRNA) targeting Rac1, the 25-nucleotide modified synthetic RNA (RAC1 Validated Stealth™ RNAi) was purchased from Invitrogen and tentatively named Rac1 siRNA number 3. The sequences were as follows: 5'-AGGGUCUAGCCAUUGCUAGGAGAU-3' (sense) and 5'-AUCUCUUGGCAUGGCUAGACCU-3' (antisense). As the following RNA, designated as Rac1 siRNA number 1, was designed for a coding region of Rac1: 5'-UCUGUGCAAAGUUGGUAUCUGAGGUA-3' (sense) and 5'-ACCUCAGGAUACCACUUGGCAAGGUAU-3' (antisense). The sequence of Rac1 siRNA number 2 corresponded to that of a 3'-untranslated region: 5'-CUUGGAAACCUCUUGGUAACCU-3' (sense) and 5'-GAGCAAACGCUAACGGAACACCCGGAA-3' (antisense). As a negative control for Rac1 siRNAs numbers 1 and 2 (cont 1), Medium GC Duplex of Stealth™ RNAi Negative Control Duplexes (Invitrogen) was used. As a negative control for Rac1 siRNA number 3 (cont 2), the following RNA was used: 5'-AGGGUCUAGCCAUUGCUAGGAGAU (sense) and 5'-AUCUCUUGGCAUGGCUAGACCU-3' (antisense). As siRNA targeting Cdc42, two different RNA duplexes (CDC42 Validated Stealth™ RNAi) were purchased from Invitrogen: 5'-CCUCUAUAUUGGAGAAACUCUUGCCA-3' (sense) and 5'-UUUGGCAAGGUUUCUCAACUUGAGGAGG-3' (antisense) as Cdc42 siRNA number 1 and 5'-UCCUCUUUCUGCUUGUUGGACUCAA-3' (sense) and 5'-UUUGAGUCCAAAGCAAGAAGGAA-3' (antisense) as Cdc42 siRNA number 2. As a negative control for Cdc42 siRNAs, Low GC Duplex of Stealth™ RNAi Negative Control Duplexes (Invitrogen) was used. The sequence of Rac3 siRNA corresponded to that of a coding region: 5'-CCUCUCUUGGAGAUAACCUUGUGGCAAGG-3' (sense) and 5'-UUUGGCAAGGAAACUCUUGGAGGAA-3' (antisense). As a negative control for Rac3 siRNA, Medium GC Duplex of Stealth™ RNAi Negative Control Duplexes (Invitrogen) was used. Cells transfected with siRNA were cultured for 48 (HeLa cells) or 72 h (Caco-2 cells), and used for estimation of protein levels and superoxide producing activities.

Two-hybrid Experiments—Various combinations between pGBT9 (Clontech) and pGADGH (Clontech) plasmids, each encoding an oxidase protein, were cotransfected into competent yeast HF7c cells containing a HIS3 reporter gene, as previously described (27, 42). Following the selection for Trp⁺ and Leu⁺ phenotype, the transformants were tested for their ability to grow on plates lacking histidine, according to the manufacturer’s recommendation (Clontech).
An in Vitro Binding Assay Using Purified Proteins—The wild-type and mutant Rac1 cDNAs were ligated to pGEX-6P (Amer- sham Biosciences) for gluthathione S-transferase (GST) fusion protein. The proteins expressed in Escherichia coli strain BL-21 (Stratagene) were purified by glutathione-Sepharose 4B (Amersham Biosciences) and cleaved with PreScission Protease (Amersham Biosciences), according to the manufacturer’s protocols. For in vitro pulldown binding assays, Rac was mixed with GST-Pak-PBD or GST alone in 400 μl of 100 mM NaCl and 20 mM Tris pH 7.6, and incubated for 30 min at 4 °C. A slurry of glutathione-Sepharose 4B was subsequently added, followed by further incubation for 60 min at 4 °C. After washing four times with 100 mM NaCl and 20 mM Tris, pH 7.6, containing 0.5% Triton X-100 and 10 mM dithiothreitool, proteins were eluted from glutathione-Sepharose 4B with 10 mM glutathione in 100 mM Tris-HCl, pH 8.0, and 200 mM NaCl. The eluates were subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue.

Estimation of Rac Activation in HeLa Cells—Rac activation in HeLa cells was estimated as previously described (55). Briefly, HeLa cells were broken by the addition of the same volume of a lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 2 mM MgCl2, 5 mM EGTA, 20 μM leupeptin, 15 μM peptatin A, 0.8 μM aprotinin, 1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 36 μM bestatin, and 14 μM cysteine protease inhibitor E64). The lysate was centrifuged for 20 s at 12,000 × g, and the supernatant was incubated with GST-Pak-PBD for 15 min in the absence or presence of GTPγS (0.1 mM) or GDPβS (1 mM). Proteins were precipitated with glutathione-Sepharose 4B (Amersham Biosciences), and the precipitants were analyzed by immunoblot with the anti-Rac antibody.

Membrane Localization of Noxa1 in HeLa Cells—Membrane fractions were prepared by the method of Leusen et al. (21, 23) with minor modifications. Briefly, transfected HeLa cells were lysed by sonication, and the sonicate (1.0 ml) was layered on a discontinuous sucrose gradient consisting of 1.5 ml of 15% (w/v) sucrose and 2.0 ml of 40% (w/v) sucrose with 1.0 mM MgCl2, 40 mM NaCl, and 0.5 mM EGTA. After ultracentrifugation for 1 h at 170,000 × g, the layer between the 15 and 40% sucrose fractions were collected and used as the membrane fraction, which were analyzed by immunoblot with the anti-HA monoclonal antibody, the anti-myct monoclonal antibody, the anti-p22phox polyclonal antibodies, or anti-integrin β1 polyclonal antibodies (Santa Cruz Biotechnology).

RESULTS

Electropermeabilized HeLa Cells, Which Ectopically Express Nox1, Noxo1, and Noxa1, Produce Superoxide in a GTP-dependent Manner—It is well known that electrically permeabilized neutrophils, which highly express gp91phox, produce superoxide in a GTP-dependent manner (60, 61). To know role of GTP-binding proteins in Nox1 activity, we tested the effect of GTP. HeLa cells, ectopically expressing Nox1 and its activating proteins Noxo1 and Noxa1, produced superoxide (data not shown), as previously shown in other types of cells (42, 50, 58). When the cells were electropermeabilized in the absence of
GTPγS, no superoxide was produced (Fig. 2A). In the presence of GTPγS, on the other hand, they produced a substantial amount of superoxide (Fig. 2A): the rate of superoxide production by permeabilized cells was about two times higher than that of intact cells. When GDPβS was used instead of GTPγS, only a negligible amount of superoxide was detected (Fig. 2B). GTPγS seems to act inside the cells, because this agent did not induce superoxide production in non-permeabilized cells (data not shown). The GTP-dependent superoxide production is likely catalyzed by Nox1, as supported by the finding that the production absolutely required expression of the three indispensable components of the Nox1-based oxidase: Nox1, Noxo1, and Noxa1 (Fig. 2C). Essentially the same results were obtained when CHO cells were used instead of HeLa cells (data not shown): in the case of CHO cells, the p22phox cDNA was simultaneously transfected, since these cells scarcely contain endogenous mRNA for p22phox; on the other hand, HeLa cells fully express the p22phox mRNA. Thus the superoxide producing activity of Nox1 appears to be controlled in a manner dependent on a GTP-binding protein.

Rac Is Involved in Nox1-dependent Superoxide Production—We have previously shown that Rac, but not Cdc42, binds to the TPR-containing region of Noxa1, and the binding is abolished when Arg-103 in the third TPR is substituted with Glu (Ref. 42; see Fig. 1). A mutant Noxa1 carrying this substitution was about 4-fold less effective in the GTP-dependent superoxide production by Nox1 than the wild-type mutant (Fig. 2C), suggesting that Rac is involved in Nox1 regulation.

To further study the role of Rac in Nox1 activation, we specifically knocked down Rac1, the predominant Rac isoform in non-hematopoietic cells, using the RNAi method, and tested its effect on Nox1-dependent superoxide production by intact cells. Transfection of HeLa cells with three different double-strand siRNA, each specific to human Rac1, led to a significant decrease in Rac at the protein level, as estimated by immunoblot analysis using an anti-Rac monoclonal antibody (Fig. 3A). On the other hand, the treatment did not affect the Noxo1 and Noxa1 proteins (Fig. 3A). The siRNA-treated cells produced a small amount of superoxide, as compared with the cells transfected with the control RNAs (Fig. 3A). When Rac1 siRNA number 2, targeted to a 3′-untranslated region, was used (see “Experimental Procedures”), superoxide production was restored by transfection of the cDNA encoding the constitutively active form of Rac1(Q61L), which lacks the 3′-untranslated region and thus mRNA derived from the cDNA is expected to be unaffected by the siRNA (Fig. 3B).

Thus the effect of the siRNA is due to a reduced level of Rac protein. To exclude the possibility that the RNAi decreases in the protein level of Nox1, we expressed Nox1 as a FLAG-tagged protein and detected the protein with an anti-FLAG antibody. As shown in Fig. 3C, the RNAi did not affect Nox1 at the protein level. We also tested the effect of the RNAi in Caco-2 cells, which endogenously express Nox1, the cells produce superoxide when both Noxo1 and Noxa1 are ectopically expressed (47). The Rac1 RNAi in Caco-2 cells resulted in an impaired production of superoxide (Fig. 3D). On the other hand, transfection of siRNA specific to Rac3 did not affect the amount of Rac protein and the superoxide producing activity in HeLa cells (data not shown). These findings indicate that endogenous Rac1 participates in Nox1 activity.

To further know the role of endogenous Rac, we studied the effect of RhoGDI, which forms a complex with Rho family GTPases including Rac and thus blocks their functions (62). Expression of RhoGDI in Caco-2 cells (Fig. 4A) and HeLa cells (Fig. 4B) inhibited Nox1-dependent production of superoxide. Similarly, the Nox1 activity was blocked by Pak-PBD (Fig. 4A), the Rac/Cdc42-binding domain of the protein kinase Pak, which is known to inhibit Rac-dependent events (63). These effects do not appear to result from the blockade of Cdc42, because knockdown of Cdc42 by RNAi did not affect the Nox1-dependent superoxide production (Fig. 4C).

We also tested the effect of ectopic expression of Rac1 as myc-tagged proteins. Rac1(Q61L) enhanced superoxide production by about 2-fold in HeLa cells containing Nox1 (Fig. 5A). On the other hand, neither a constitutively active Cdc42(Q61L) (Fig. 5B) nor RhoA(Q63L) (data not shown) could facilitate Nox1-dependent superoxide production. In addition, a dominant negative form of Rac1(T17N) as well as the wild-type Rac1 was incapable of activating Nox1 (Fig. 5A). Under the conditions, the ratio of ectopically expressed Rac to endogenous Rac was 1:2 in transfected HeLa cells, as estimated by immunoblot analysis using the anti-Rac antibody. Low expression levels of the
Mechanism for Rac-mediated Regulation of Nox1

transfected proteins relative to endogenous Rac levels may, in part, explain the reason why Rac(T17N) fails to inhibit the Nox1 activity (Fig. 5A). The present findings thus indicate that Rac in the GTP-bound active form specifically participates in Nox1 activation.

Since Nox1 produced a substantial amount of superoxide without expression of Rac(Q61L) (Fig. 5A), it seems possible that endogenous Rac is at least partially active in unstimulated HeLa cells. To test this possibility, we pulled down the active GTP-bound form of Rac from the cell lysate using GST-Pak-PBD by a previously described method (57). As shown in Fig. 5C, a small but significant amount of Rac was precipitated, indicating that a part of Rac is in the active form in HeLa cells.

Interaction of Rac with Noxa1 Participates in Nox1 Activation—The observation that the mutant Noxa1(R103E) is much less effective in Nox1 activation than the wild-type Noxa1 (Fig. 2C) suggests that Rac functions via interacting with Noxa1. In addition, Rac1(Q61L) was incapable of activating Nox1 in the absence of Noxa1 (data not shown). To confirm the role of the Rac-Noxa1 interaction, we tested the ability of mutant Rac proteins to support superoxide production by Nox1.

It has been reported that A27K substitution in Rac results in a loss of the interaction with p67phox, although it does not seem to affect the ability to activate the protein kinase Pak, a molecule involved in a Cdc42/Rac-mediated signaling pathway (64). As expected, a GTPase-deficient active Rac with this substitution (A27K/Q61L) only marginally activated gp91phox/Nox2 in HeLa cells expressing p67phox and p47phox (Fig. 5D). Rac is considered to recognize Noxa1 in a way similar to that for p67phox, because Rac binding is abrogated by the substitution of Glu for Arg-102 of p67phox (27, 50), which corresponds to Arg-103 of Noxa1. Consistent with this, the mutant Rac1 carrying the A27K substitution failed to interact well with Noxa1 in the yeast two-hybrid system (Fig. 5E), but fully interacted with Pak (Fig. 5F). In addition, Rac1(A27K/Q61L) was incapable of enhancing superoxide production by Nox1 in HeLa cells (Fig. 5A), indicative of the role for the Rac-Noxa1 interaction.

It is also known that, although a mutant Rac1 carrying the Y40C substitution is incapable of binding to Pak and thus fails

FIGURE 5. Effect of expression of the active Rac mutants on Nox1-dependent superoxide production. A, B, and D, HeLa cells were cotransfected with the indicated plasmids: pcDNA3.0-Nox1, pEF-BOS-HA-Noxo1, pEF-BOS-myc-Noxa1, pcDNA3.0-gp91phox/Nox2, pEF-BOS-HA-p47phox, pEF-BOS-myc-p67phox, pEF-BOS-myc-Rac1(wt), pEF-BOS-myc-Rac1(Q61L), pEF-BOS-myc-Rac1(A27K/Q61L), pEF-BOS-myc-Rac1(Y40C/Q61L), pEF-BOS-myc-Rac1(T17N), pEF-BOS-myc-Cdc42(Q61L), and/or pEF-BOS-myc-Cdc42(T17N). After preincubation of the transfected, unpermeabilized cells for 5 min, superoxide production was assayed by chemiluminescence using DIOGENES. Each graph represents the mean ± S.D. of the chemiluminescence values integrated for 10 min, which were obtained from three independent transfections. Protein levels of the indicated proteins were estimated by immunoblot analysis with the anti-HA or anti-myc monoclonal antibody.

C, HeLa cells were lysed, and proteins in the lysate were precipitated in the presence or absence of GTP·S (0.1 mM) or GDP·S (1 mM) with GST-Pak-PBD bound to glutathione-Sepharose 4B beads. Rac in the precipitant or the whole cell lysate was estimated by immunoblot analysis with the anti-Rac monoclonal antibody. The experiments have been repeated more than three times with similar results.

E, interaction of the active Rac mutants with Noxa1 was estimated by the yeast two-hybrid system. The yeast HF7c cells were cotransformed with recombinant plasmids pGBT9 encoding Rac1(Q61L), Rac1(A27K/Q61L), Rac1(Y40C/Q61L), or Rac1(T17N) and pGADGH encoding the N terminus of Noxa1. Following the selection for Trp+ and Leu+ phenotype, its histidine dependent (right) and independent (left) growth was tested as described under “Experimental Procedures.” F, GST alone or GST-Pak were incubated with the indicated form of Rac1, and pulled down with glutathione-Sepharose 4B. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. These experiments have been repeated more than three times with similar results.
Mechanism for Rac-mediated Regulation of Nox1

Since Noxa1 directly binds to Nox1 via the Noxa1 SH3 domain (42), we investigated the role of the Noxa1-Noxo1 interaction in Nox1 activation. As shown in Fig. 7A, in the absence of Noxo1, Noxa1 did not localize to the membrane fraction that contained the membrane marker protein integrin β1 and p22phox. On the other hand, expression of Noxo1 elicited membrane localization of Noxa1 in a dose-dependent manner (Fig. 7A). We next used a mutant Noxa1 carrying the W436R substitution in the SH3 domain, which results in a defective binding to Noxo1 (42). Noxa1(W436R) could not support superoxide production by Nox1 (Fig. 7B) or localize to the membrane (Fig. 7C) in the presence of Noxo1. Under these conditions, the wild-type Noxa1 and Noxa1(R103E) were targeted to the membrane (Fig. 7C). Thus Noxa1 binding to Noxo1 is required for membrane recruitment of Noxa1, which plays a crucial role in Nox1 activation.

Taken together, the present findings show that Noxa1 function requires its association with the membrane, which is mediated via binding of Noxa1 to Noxo1, a protein that is directly tethered to the Nox1 partner p22phox at the membrane.

The Rac-Noxa1 Interaction Is Involved in Membrane Recruitment of Nox1 in the Absence of the Noxa1-Noxo1 Interaction—We next tested the role of Rac in membrane recruitment of Noxa1, which is crucial for Nox1 activation. The recruitment does not seem to require Rac in the presence of Noxo1, since it was not affected by depletion of Rac by RNAi (Fig. 8A). However, in the absence of Noxo1, membrane localization of Noxa1 was totally dependent on expression of Rac1(Q61L) (Fig. 8B). Rac likely functions by binding to Noxa1, because Noxa1(R103E), deficient in binding to Rac, failed to move to the membrane even in the presence of Rac1(Q61L) (Fig. 8B).

Although Noxa1(W436R), incapable of interacting with Nox1, did not localize to the membrane in the absence of Rac1(Q61L) (Fig. 7C), expression of Rac1(Q61L) caused membrane association of Noxa1(W436R) to the same extent as that of the wild-type Noxa1 (Fig. 7D). Thus active Rac has the ability to fully recruit Noxa1 to the membrane even without the Noxa1-Noxo1 interaction. In parallel with this, when Rac1(Q61L) was co-expressed, Noxa1(W436R) supported Noxa1 activation as much as the wild-type protein (Fig. 7B). By contrast, a doubly mutated Noxa1 with R103E/W436R substitution, which can bind neither Rac nor Nox1, was incapable of associating with the membrane (Fig. 7D) or supporting Noxa1 activation (Fig. 7B) even in the presence of Rac1(Q61L). These findings indicate that Rac directly binds to Noxa1 and thus

FIGURE 6. Role of interaction between Noxo1 and p22phox on Nox1-dependent superoxide production. CHO cells (A) or HeLa cells (B) were cotransfected with pCDNA3.0- Nox1 and pEF-BOS-myc-Noxa1 and simultaneously with the indicated plasmids: the pEF-BOS vector, pEF-BOS-p22phox(wt), pEF-BOS-p22phox(P156Q), pEF-BOS-myc-Rac1(Q61L), pEF-BOS-HA-Noxo1(wt), and/or pEF-BOS-HA-Noxo1(W197R). After preincubation of the transfected, unpermeabilized cells for 5 min, superoxide production was assayed by chemiluminescence using DIOGENES. Each graph represents the mean ± S.D. of the chemiluminescence values integrated for 10 min, which were obtained from three independent transfections. Protein levels of the indicated proteins were estimated by immunoblot analysis with the anti-p22phox polyclonal, anti-myc monoclonal, or anti-HA monoclonal antibodies.
facilitates membrane recruitment of this protein, an effect that is cryptic in the presence of Noxa1-Noxo1 interaction, and that the Rac-mediated recruitment plays a crucial role in Nox1 activation. However, Noxa1 recruitment by itself is not enough for Nox1 activation: in the absence of Noxa1, expression of Rac1(Q61L) led to membrane targeting of Noxa1 (Fig. 8C) but did not induce superoxide production (data not shown). These findings also suggest that the role of Noxa1 is not restricted to membrane recruitment of Noxa1.

It is known that Rac localizes to the membrane via the geranylgeranyl group linked to Cys-189 (66). The C189S substitution in Rac1, leading to a loss of the modification, resulted in an impaired membrane association (Fig. 8, C–E). Mutant Rac1(Q61L/C189S) failed to elicit membrane recruitment of the wild-type Noxa1 in the absence of Noxo1 (Fig. 8C) or that of Noxa1(W436R) in the presence of Noxo1 (Fig. 8D). Thus membrane localization of Rac is required for Rac-dependent Noxa1 recruitment.

Rac-Noxa1 Interaction Plays a Role in Nox1 Activation at the Membrane—Although the mutant Noxa1(R103E) was fully recruited to the membrane (Fig. 6B), it failed to support superoxide production by Nox1, indicating that Rac functions with Noxa1 at the membrane. This is supported by the finding that the mutant Rac1(Q61L/C189S), defective in membrane targeting, was incapable of enhancing Nox1 activity (Fig. 8E). In activation of gp91(phox)/Nox2, Noxa1 as well as p67(phox) functions not only by interacting with Rac but also by using the activation domain C-terminal to the Rac-binding TPR domain (Fig. 1): V205A substitution in the activation domain leads to an impaired activation of gp91(phox) (50, 67). In the case of p67(phox), Rac binding to the TPR domain is considered to induce a conformational change of the activation domain, leading to gp91(phox) activation (12–14). The mutant Noxa1(V205A) as well as Noxa1(R103E) was inactive in Nox1 activation in HeLa cells (Fig. 9A) and Caco-2 cells (Fig. 9B), indicating a crucial role of the Nox1 activation domain. Importantly, Noxa1(V205A) was fully targeted to the membrane in a manner independent of the presence of Rac1(Q61L) (Fig. 9, C and D). Thus Rac appears to function in Nox1 activation via rendering the Noxa1 activation domain in a functional state.

**DISCUSSION**

In the present study, we show that the small GTPase Rac is directly involved in activation of Nox1. Rac appears to function by interacting with the oxidase activator Noxa1. The Rac-Noxa1 interaction at the membrane plays a crucial role in Nox1 activation, which possibly induces a conformational change of the activation domain of Noxa1. Membrane recruitment of Noxa1, an event that is crucial for Nox1 activation, is normally dependent on association of Noxa1 with the oxidase organizer.
In the absence of sufficient interaction between Noxo1 and Noxa1, the recruitment requires the binding of Rac to Noxa1, suggesting that Rac functions partly by facilitating membrane localization of Noxa1.

The role of Rac in Nox1 activation has been suggested by previous observations: blockade of the conversion of Rac to the GTP-bound state inhibits Nox1-dependent superoxide production in guinea pig gastric mucosal cells, in which inhibition is restored by expression of Rac1(Q61L) (54); and both Rac activation and Nox1-dependent ROS production are downregulated in cells depleted of the Rac activator βPix by RNAi (53). The involvement of Rac is also supported by the present finding that superoxide production by HeLa cells, ectopically expressing Nox1, Noxo1, and Noxa1, or by Caco-2 cells containing endogenous Nox1 is significantly decreased when Rac is depleted by RNAi-mediated gene silencing (Fig. 3) or sequestered by RhoGDI or Pak-PBD (Fig. 4). In addition, a part of Rac in unstimulated HeLa cells appears to be in the active form (Fig. 5), which may explain at least partially the reason why Nox1 constitutively produces superoxide. On the other hand, it has remained obscure whether Rac is directly involved in the active, fully assembled Nox1 complex, as in the phagocyte oxidase complex, or indirectly activates Nox1 via acting in an upstream signaling pathway, e.g. a Pak-containing pathway.

The present study demonstrates that Rac directly participates in formation of the active Nox1 complex via binding to Noxa1. Superoxide production by Nox1 in electrically permeabilized HeLa cells is entirely dependent on GTP (Fig. 2). The production is abrogated when the mutant Noxa1(R103E), defective in binding to Rac, is expressed instead of the wild-type one. Furthermore, expression of the constitutively active Rac1(Q61L) leads to enhancement of Nox1-dependent superoxide production, the enhancement is not observed with the mutant Rac1(A27K/Q61L), defective in binding to Rac, is expressed instead of the wild-type one. Furthermore, although expression of the constitutively active Rac1(Q61L) leads to enhancement of Nox1-dependent superoxide production, the enhancement is not observed with the mutant Rac1(A27K/Q61L), defective in binding to Noxa1 (Fig. 5). In addition, the mutant Rac1(Y40C/Q61L), which can bind to Noxa1 but not to Pak, enhances the Nox1 activity (Fig. 5), supporting the conclusion that Rac is directly involved in Nox1 activation rather than via a Pak-containing signaling pathway. It has been reported that Rac can directly bind to the C-terminal region of Nox1 in a GTP-dependent manner (53). This binding may function to activate Nox1 in cooperation with the Rac-Noxa1 interaction. During the course of the revision of our paper, two other groups have also shown the direct role of Rac in Nox1 activation (68, 69).

The Rac-Noxa1 interaction appears to serve at two distinct steps: support of membrane recruitment of Noxa1 and induction of a conformational change of Noxa1 for Nox1 activation.
Mechanism for Rac-mediated Regulation of Nox1

For both steps, membrane targeting of Rac appears to be required, because Rac1(Q61L)/C189S, defective in membrane localization, fails to recruit Nox1 to the membrane and to support superoxide production (Fig. 8).

Membrane recruitment of Noxa1 appears to act as a crucial step for activation of Nox1, as shown for the first time in the present study. Noxa1 seems to reside on the membrane primarily via interacting with Noxo1; Noxa1 fails to associate with the membrane in the absence of Noxo1. The interaction is mediated by binding of the Noxa1 SH3 domain to the Noxo1 C terminus (Ref. 42; see Fig. 1). When the interaction is disrupted by the W436R substitution in the Noxa1 SH3 domain (Ref. 42; see Fig. 1), protein levels in the membrane fraction of cells with Rac1(Q61L) were analyzed by immunoblot with the anti-Rac, anti-HA, or anti-myc monoclonal antibody, and also with the anti-p22phox or anti-integrin β1 (a membrane marker protein) polyclonal antibodies as a loading control. Caco-2 cells were cotransfected with pEF-BOS-HA-Noxo1, and pEF-BOS-myc-Noxa1(wt), pEF-BOS-myc-Noxa1(R103E), pEF-BOS-myc-Noxa1(V205A), or pEF-BOS-myc-Noxa1(W436R). Supernoxide production and protein levels of whole cell lysate were estimated as described in A. These experiments have been repeated more than three times with similar results.

FIGURE 9. Effect of the V205A substitution of Noxa1 on Noxa1 membrane localization and Nox1-dependent superoxide production. A, C, and D, HeLa cells were cotransfected with the indicated plasmids: pcDNA3.0-Nox1, pEF-BOS-HA-Noxo1, pEF-BOS-myc-Noxa1(wt), pEF-BOS-myc-Noxa1(V205A), pEF-BOS-myc-Noxa1(R103E), and/or pEF-BOS-myc-Rac1(Q61L). After preincubation of the transfected, impermeabilized cells for 5 min, superoxide production was assayed by chemiluminescence using DIOGENES: each graph represents the mean ± S.D. of the chemiluminescence values integrated for 10 min, which were obtained from three independent transfections (A). The whole cell lysate was analyzed by immunoblot with the anti-HA or anti-myc monoclonal antibody (A). Protein levels in the membrane fraction of cells with (D) or without (C) Rac1(Q61L) were analyzed by immunoblot with the anti-Rac, anti-HA, or anti-myc monoclonal antibody, and also with the anti-p22phox or anti-integrin β1 (a membrane marker protein) polyclonal antibodies as a loading control. B, Caco-2 cells were cotransfected with pEF-BOS-HA-Noxo1, and pEF-BOS-myc-Noxa1(wt), pEF-BOS-myc-Noxa1(R103E), pEF-BOS-myc-Noxa1(V205A), or pEF-BOS-myc-Noxa1(W436R). Supernoxide production and protein levels of whole cell lysate were estimated as described in A. These experiments have been repeated more than three times with similar results.

AUGUST 4, 2006

For both steps, membrane targeting of Rac appears to be required, because Rac1(Q61L)/C189S, defective in membrane localization, fails to recruit Nox1 to the membrane and to support superoxide production (Fig. 8).

Membrane recruitment of Noxa1 appears to act as a crucial step for activation of Nox1, as shown for the first time in the present study. Noxa1 seems to reside on the membrane primarily via interacting with Noxo1; Noxa1 fails to associate with the membrane in the absence of Noxo1. The interaction is mediated by binding of the Noxa1 SH3 domain to the Noxo1 C terminus (Ref. 42; see Fig. 1). When the interaction is disrupted by the W436R substitution in the Noxa1 SH3 domain, both Noxa1 membrane localization and Nox1-dependent superoxide production are abrogated (Fig. 7). On the other hand, Nox1 is tethered to p22phox, the membrane partner of Nox1, via the SH3-mediated association (Ref. 42; see Fig. 1), which is required for Nox1 activation (Fig. 6). Thus Noxa1 is linked by Nox1 to the membrane. In the presence of sufficient interac-

tion between Noxa1 and Noxo1, the requirement of Rac for Noxa1 membrane recruitment is cryptic, although Rac is still necessary for Nox1 activation (Fig. 7). On the other hand, once the Noxa1-Noxo1 interaction is disrupted, both membrane targeting of Noxa1 and activation of Nox1 become largely dependent on active Rac (Fig. 7).

This indicates an important role of Rac, and also raises the possibility that the balance between Noxa1 and Noxo1 determines the extent of Rac contribution. It is known that the expression pattern of Noxa1 is different from that of Nox1 (42, 46, 47). A role for Rac in membrane recruitment of p67phox has been shown under cell-free conditions: when excessive amounts of Rac and p67phox are present, activation of gp91phox/Nox2 is possible without p47phox in a cell-free system, suggestive of the role for Rac (70, 71); and Rac indeed recruits p67phox to the membrane prepared from macrophages in vitro (35).

In addition to the step for membrane recruitment of Nox1, Rac appears to play another direct role in Nox1 activation: Noxa1(R103E), defective in binding to Rac, is incapable of activating Nox1, although it is fully targeted to the membrane (Fig. 7). Rac likely binds to Noxa1 in a manner similar to that for the binding to p67phox, because Rac fails to bind to a mutant p67phox with substitution of Glu for Arg-102 (27), in which the residue corresponds to Arg-103 of Noxa1. In addition, Rac1(Y40C), defective in binding to Pak, is capable of binding to Noxa1 (Fig. 5) as well as p67phox (65), whereas Rac1(A27K) interacts with neither Noxa1 nor p67phox. Since the Rac-p67phox interaction is considered to induce a conformational change of the p67phox activation domain, leading to activation of gp91phox/Nox2 (14, 33–35), it seems possible that Noxa1 also undergoes a conformational change of its activation domain for Nox1 activation, via binding to Rac. Consistent with this, a mutant Noxa1 carrying the substitution of Ala for Val-205 in the activation domain is inactive in Nox1 activation, although it is fully localized to the membrane (Fig. 9).

Rac is thus involved in Nox1 activation; however, it is presently unclear whether this GTPase is absolutely required. Even when most of Rac (more than 90%) is depleted by RNAi, the siRNA-treated cells produce about half an amount of superoxide compared with untreated cells (Fig. 3). In addition, Noxa1(R103E) exerts a weak but significant effect on Nox1 acti-
Mechanism for Rac-mediated Regulation of Nox1

26. Mizuki, K., Takeya, R., Kuribayashi, F., Nobuhisa, I., Kohda, D., Nuno, H., Takeshige, K., and Sumimoto, H. (2005) Arch. Biochem. Biophys. 444, 185–194
27. Koga, H., Terasawa, H., Nuno, H., Takeshige, K., Inagaki, F., and Sumimoto, H. (1999) J. Biol. Chem. 274, 25051–25060
28. Lapouge, K., Smith, S. J. M., Walker, P. A., Gamblin, S. J., Smerdon, S. J., and Rittering, K. (2000) Mol. Cell 6, 899–907
29. Price, M. O., McPhail, L. C., Lambeth, J. D., Han, C.-H., Knaus, U. G., and Dinauer, M. C. (2002) Blood 99, 2653–2661
30. Biberstein-Kinkade, K. J., Yu, L., Stull, N., LeRoy, B., Bennett, S., Cross, A., and Dinauer, M. C. (2002) J. Biol. Chem. 277, 30368–30374
31. Miyano, K., Ogasa, S., Han, C. H., Fukuda, H., and Tamura, M. (2001) Biochemistry 40, 14089–14097
32. Hata, K., Takeshige, K., and Sumimoto, H. (1997) Biochem. Biophys. Res. Commun. 241, 226–231
33. Han, C.-H., Freeman, I. L. R., Lee, T., Motolebi, S. A., and Lambeth, J. D. (1998) J. Biol. Chem. 273, 16663–16668
34. Aloul, N., Gorzalczyzny, Y., Itan, M., Sigal, N., and Pick, E. (2001) Biochemistry 40, 14557–14566
35. Gorzalczyzny, Y., Aloul, N., Sigal, N., Weinbaum, C. K., and Pick, E. (2002) J. Biol. Chem. 277, 18605–18610
36. Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., Chung, A. B., Gregoire, K. J., and Lambeth, J. D. (1999) Nature 401, 79–82
37. Bánfi, B., Maturana, A., Jaconi, S., Arnaudese, S., Laforge, S., Sinha, B., Ligeti, E., Demaures, N., and Krause, K.-H. (2000) Science 287, 138–142
38. Geiszt, M., Lekstrom, K., Brenner, S., Hewitt, S. M., Dana, R., Malech, H. L., and Leto, T. L. (2003) J. Immunol. 171, 299–306
39. Kawahara, T., Kuwano, Y., Teshima-Kondo, S., Takeya, R., Sumimoto, H., Kishi, K., Tsunawaki, S., Hiyama, T., and Rokutan, K. (2004) J. Immunol. 172, 3051–3058
40. Matsuno, K., Yamada, H., Iwata, K., Jin, D., Katsuyama, M., Matsuki, M., Takai, Y., Yanamishi, K., Miyazaki, M., Matsubara, H., and Yabe-Nishimura, C. (2005) Circulation 112, 2677–2685
41. Dikalova, A., Ciemrus, R., Lassegue, B., Cheng, G., McCoy, J., Dikalov, S., San Martin, A., Lyle, A., Weber, D. S., Weiss, D., Taylor, W. R., Schmidt, H. H., Owens, G. K., Lambeth, J. D., and Greifeld, K. K. (2005) Circulation 112, 2668–2676
42. Takeya, R., Ueno, N., Komi, K., Taura, M., Kohjima, M., Izaki, T., Nuno, H., and Sumimoto, H. (2003) J. Biol. Chem. 278, 25234–25246
43. Amba, R. K., Kumar, P., Gregoire, K. K., Schmidt, H. H., Busse, R., and Brandes, R. P. (2004) J. Biol. Chem. 279, 45935–45941
44. Hanna, I. R., Hilenski, L. L., Dikalov, S., Taniyama, Y., Smerdon, S. J., and Takeya, R., S, 2002, 144, 2329–2339
45. Martin, K. D., Frederick, L. M., von Loehneysen, K., Dinauer, M. C., and Krause, K. U. (2006) Cell Signal. 18, 69–82
46. Fortemaunon, N., Miot, F., Dumont, J. E., and Dervieu, S. (2005) Eur. J. Endocrinol. 152, 127–133
47. Park, H. S., Lee, S. H., Park, D., Lee, J. S., Ryu, S. H., Lee, W. J., Rhee, S. G., and Bae, Y. S. (2004) Mol. Cell 20, 2328–2339
48. Akasaka, T., Koga, H., and Sumimoto, H. (1999) J. Biol. Chem. 274, 20805–20809
49. Leffers, H., Nielsen, M. S., Andersen, A. H., Honore, B., Madsen, P., Vandestekkhove, J., and Celis, J. E. (1993) Exp. Cell Res. 209, 165–174
Mechanism for Rac-mediated Regulation of Nox1

57. Mizushima, S., and Nagata, S. (1990) Nucleic Acids Res. 18, 5322
58. Takeya, R., Ueno, N., and Sumimoto, H. (2005) Methods Enzymol. 406, 456–468
59. Grinstein, S., and Furuya, W. (1988) J. Biol. Chem. 263, 1779–1783
60. Nasmith, P. E., Mills, G. B., and Grinstein, S. (1989) Biochem. J. 257, 893–897
61. Tamura, M., Yoshida, K., and Kataoka, K. (1999) Arch. Biochem. Biophys. 361, 257–263
62. Lu, W., and Mayer, B. J. (1999) Oncogene 18, 797–806
63. Price, L. S., Leng, J., Schwartz, M. A., and Bokoch, G. M. (1998) Mol. Biol. Cell 9, 1863–1871
64. Kwong, C. H., Adams, A. G., and Leto, T. L. (1995) J. Biol. Chem. 270, 19868–19872
65. Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) Cell 87, 519–529
66. Kreck, M. L., Freeman, J. L., Abo, A., and Lambeth, J. D. (1996) Biochemistry 35, 15683–15692
67. Cheng, G., Ritsick, D., and Lambeth, J. D. (2004) J. Biol. Chem. 279, 34250–34255
68. Ueyama, T., Geiszt, M., and Leto, T. L. (2006) Mol. Cell. Biol. 26, 2160–2174
69. Cheng, G., Diebold, B. A., Hughes, Y., and Lambeth, J. D. (April 24, 2006) J. Biol. Chem. 281, 10.1074/jbc.M512751200
70. Freeman, J. L., and Lambeth, J. D. (1996) J. Biol. Chem. 271, 22578–22582
71. Koshkin, V., Lotan, O., and Pick, E. (1996) J. Biol. Chem. 271, 30326–30329