The NADPH Oxidase Nox3 Constitutively Produces Superoxide in a p22phox-dependent Manner

ITS REGULATION BY OXIDASE ORGANIZERS AND ACTIVATORS*

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Nox3, a member of the superoxide-producing NADPH oxidase (Nox) family, participates in otocionia formation in mouse inner ears, which is required for perception of balance and gravity. The activity of other Nox enzymes such as gp91phox/Nox2 and Nox1 is known to absolutely require both an organizer protein (p47phox or Nox1) and activator protein (p67phox or Nox1a); for the p47phox-dependent activation of these oxidases, treatment of cells with stimulants such as phorbol 12-myristate 13-acetate is also indispensable. Here we show that ectopic expression of Nox3 in various types of cells leads to phorbol 12-myristate 13-acetate-dependent constitutive production of a substantial amount of superoxide under the conditions where gp91phox and Nox1 fail to generate superoxide, i.e. in the absence of the oxidase organizers and activators. Nox3 likely forms a functional complex with p22phox; Nox3 physically interacts with and stabilizes p22phox, and the Nox3-dependent superoxide production is totally dependent on p22phox. The organizers p47phox and Nox1a are capable of enhancing the superoxide production by Nox3 in the absence of the activators, and the enhancement requires the interaction of the organizers with p22phox, further indicating a link between Nox3 and p22phox. The p47phox-enhanced Nox3 activity is further facilitated by p67phox or Nox1a, whereas the activators cancel the Nox1-induced enhancement. On the other hand, the small GTPase Rac, essential for the gp91phox activity, is likely dispensable to the Nox3 system. Thus Nox3 functions together with p22phox as an enzyme constitutively producing superoxide, which can be distinctly regulated by combinatorial use of the organizers and activators.

It is currently recognized that in a variety of cells reactive oxygen species (ROS) are produced not only as by-products in aerobic metabolism but also as true products by specialized enzymes to play roles in various events such as host defense, oxygen sensing, and signal transduction (1–7). Such ROS-generating enzymes include members of the superoxide-producing NADPH oxidase (Nox) family, which contain heme-binding sites on the membrane-spanning region in the N-terminal half and the NADPH- and FAD-binding domains in the C-terminal half, thereby forming a complete electron-transporting apparatus from NADPH to molecular oxygen (Refs. 1–7; for Nox3, see Fig. 1A).

The prototype Nox enzyme gp91phox, also termed Nox2, is predominantly expressed in professional phagocytes, such as neutrophils and macrophages, and tightly associates with the membrane-integrated protein p22phox to form cytochrome b556; the association stabilizes both proteins (5–7). gp91phox is dormant in resting cells but becomes activated during phagocytosis to produce superoxide, a precursor of microbicidal ROS. The activation of gp91phox requires the two specialized proteins p47phox and p67phox, each containing two SH3 domains, and the small GTPase Rac (Fig. 1B). These proteins exist in the cytoplasm of resting phagocytes and translocate upon cell stimulation to the membrane to interact with cytochrome b556, which allows gp91phox to transport electrons for superoxide production (8–13). The significance of the phagocyte oxidase system in host defense is exemplified by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD) whose phagocytes lack the superoxide-producing activity (14, 15).

The membrane recruitment of p47phox and subsequent activation of gp91phox require the interaction of the p47phox SH3 domains with the proline-rich region (PRR) in the C terminus of p22phox, the partner of gp91phox (Refs. 16 and 17; see Fig. 1B). A mutant p47phox carrying the W193R substitution in the N-terminal SH3 domain neither binds to p22phox nor activates gp91phox (18, 19), and the substitution of Gln for Pro-156 in the PRR of p22phox (P156Q), a mutation found in a patient with CGD (20, 21), leads to a defective binding to p47phox (16, 17). In resting cells, the SH3 domains of p47phox are masked via an intramolecular interaction with the autoinhibitory region (AIR) that locates C-terminal to the SH3 domains (22–25). Upon cell stimulation, p47phox undergoes phosphorylation on multiple serines in the AIR (26–30), which induces a conformational change to render the SH3 domains in a state accessible to p22phox (22, 28, 29). Thus the regulated interaction between p47phox and p22phox has been proposed to control the superoxide production in phagocytes (16, 17)

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‡ The abbreviations used are: ROS, reactive oxygen species; Nox, NADPH oxidase; CGD, chronic granulomatous disease; PRR, proline-rich region; AIR, autoinhibitory region; PMA, phorbol 12-myristate 13-acetate; TPR, tetratricopeptide repeat; Noxo1, Nox organizer 1; Noxa1, Nox activator 1; GST, glutathione S-transferase; SH, Src homology; CHO, Chinese hamster ovary; HEK, human embryonic kidney; HA, hemagglutinin.
p47phox and p22phox functions as a switch for the gp91phox activation. The phosphorylation of p47phox and subsequent superoxide production by gp91phox can be induced by treatment of cells with phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C, and the induction is prevented by protein kinase C inhibitors (31, 32).

On the other hand, p67phox translocates to the membrane in a manner dependent on its binding to p47phox, which is mediated via a tail-to-tail interaction between the C-terminal SH3 domain of p67phox and the C-terminal PRR of p47phox (Refs. 33–35; see Fig. 1B). At the membrane, p67phox directly interacts with GTP-bound Rac via the N-terminal region of ~200 amino acid residues, containing four tetratricopeptide repeat (TPR) motifs (36, 37). A mutant p67phox carrying the substitution of Glu for Arg-102 in the third TPR fails to interact with Rac and is incapable of supporting superoxide production by gp91phox (36). In addition, the deletion of a region C-terminal to the Rac-binding domain in p67phox (amino acid residues 200–212), a so-called activation domain, or the V204A substitution (TPR) motifs leads to a lack of superoxide production by gp91phox (38, 39). Thus p67phox activates gp91phox both by interacting with Rac via the TPR domain (36) and by using the activation domain (38, 39).

Nox1 is abundant in colon epithelial cells and vascular smooth muscle cells (40, 41) and has been suggested to participate in host defense at the colon (42) and signal transduction leading to hypertrophy and angiogenesis (40, 43). This Nox protein is also inactive without the SH3-harboring regulatory proteins. Activation of Nox1 occurs in the presence of both Nox1 (Nox organizer 1) and Noxa1 (Nox activator 1), novel respective homologues of p47phox and p67phox, even without cell stimulants such as PMA (44–47). Nox1 can also be activated by p47phox and p67phox but to a lesser extent, whereas these classical homologues are preferable to the novel ones in activation of gp91phox (46). The novel organizer Noxo1, also known as p41nox (45, 46), has the same domain architecture as that of the classical organizer p47phox, except for the absence of an AIR (Refs. 44–46; see Fig. 1C). As a result, Nox1 is capable of binding to p22phox even in a resting state (46), which seems to explain why cell treatment with PMA is dispensable to the Nox1-dependent superoxide-producing activity of Nox1, but essential for the p47phox-dependent one (46). The novel activator Noxa1, also known as p51nox (45, 46), can bind to GTP-bound Rac via the N-terminal TPR domain (46). Because substitution of Ala for Val-205 of Noxa1, corresponding to Val-204 of the classical activator p67phox, results in a loss of superoxide production by gp91phox (48), the activation domain of Noxa1 may be also involved in the gp91phox activation; however its role in the Nox1 activity remains unclarified.

Nox3 has been initially identified as the third oxidase abundant in the human fetal kidney (49, 50). This oxidase has been recently shown to be highly expressed in the inner ear of mouse and involved in formation of otoconia, tiny mineralized structures, which is required for perception of balance and gravity; mouse mutations in Nox3 result in a head tilt phenotype because of a lack of otoconia (51). Two groups of investigators have very recently reported Nox3 regulation by oxidase organizers and activators (48, 52). Cheng et al. (48) have demonstrated that Nox3 is inactive in the absence of the organizers and activators but can be activated by Noxo1 alone and also by p67phox alone, but to a lesser extent; the p67phox-dependent activation is further enhanced by p47phox. On the other hand, Bánfi et al. (52) have shown that Nox3 on its own generates low levels of ROS in a PMA-dependent manner, whereas full activation of Nox3 requires both an organizer and an activator. Thus the effects of the regulatory proteins on the Nox3 activity are somewhat controversial. In addition, whether p22phox participates in the Nox3 system has remained to be elucidated. The role of Rac in the Nox3-dependent superoxide production has also remained unknown.

In the present study, we show that ectopic expression of Nox3 in a variety of cells leads to superoxide production without cell stimulants such as PMA. The superoxide-producing activity of Nox3 absolutely requires p22phox and Nox3 is coimmunoprecipitated with p22phox, indicating that Nox3 forms a functional complex with p22phox. The oxidase organizers and activators, each being dispensable for the Nox3 activity, can modulate the superoxide production by Nox3. In the absence of the activators, p47phox and Noxo1 can enhance the Nox3 activity, which requires the interaction of the organizers with p22phox, further supporting a link between Nox3 and p22phox. The p47phox-induced enhancement can be facilitated by p67phox and Noxa1, whereas these activators have an ability to suppress the Noxo1-mediated enhancement. In contrast to these possible regulatory roles of the organizers and activators, Rac does not appear to be involved in the Nox3 activity.

EXPERIMENTAL PROCEDURES

Chemicals—PMA was purchased from Research Biochemicals International, diphenyleneiodonium chloride was purchased from Sigma, and GF109203X was purchased from Biomol Research Laboratories. Other chemicals used were of the highest purity commercially available.

Plasmid Construction—The cDNA for human Nox3 was cloned by PCR, using a human fetal kidney cDNA library (Clontech) as a template with two specific primers, 5'-CGAGATCATGAGGTGGTGCAGATTGTT-3' (forward primer) and 5'-CGTGAATTCAGAGCTCTCTTGGTAGTAT-3' (reverse primer). The human Nox3 cDNA obtained encodes the full-length protein containing 568 amino acid residues, the sequence of which is identical to that previously reported by Cheng et al. (50). The human cDNAs encoding gp91phox/Nox2, Nox1, p22phox, p47phox, p67phox, Noxo1, Noxa1, a dominant negative form of Rac with the T17N substitution, and an acutely active form of Rac with the Q61L substitution were prepared as described previously (36, 46). The cDNA fragment for the tandem SH3 domains of Nox1 (amino acids 154–292), Noxo1-(SH3)p, was constructed by PCR using the human Nox1 cDNA. Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. The DNA fragments were ligated to the indicated expression vectors. All the constructs were sequenced to confirm their identities.

Transfection of cDNAs Encoding Nox3, gp91phox, p22phox, and Cytosolic Regulatory Proteins in COS-7, Human Embryonic Kidney (HEK293), HeLa, or CHO Cells—The cDNAs for Nox3 and gp91phox were ligated to the mammalian expression vector pcDNA3.0 (Invitrogen). The cDNA encoding p22phox, p47phox, p67phox, Noxo1, and Noxa1 were ligated to the mammalian expression vector pEF-BOS (53). p47phox and Noxo1 were constructed for expression as an HA-tagged protein, p67phox and Noxa1 as a Myc-tagged protein, and p22phox as a protein without a tag. Transfection of the monkey kidney COS-7 cells and HeLa cells with the cDNAs was performed using Lipofectamine (Invitrogen), whereas FuGENE 6 transfection reagent (Roche Applied Science) was used for transfection of the Chinese hamster ovary CHO cells and the HEK293 cells.

Superoxide-producing Activity of Cells Expressing Nox3 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 Heps, pH 7.4), the cells were tested for estimation of the superoxide-producing activity. The activity was determined by superoxide dismutase-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (DIODE- NES, National Diagnostics), as described previously (30, 36, 46). After the addition of the enhanced luminol-based substrate, the cells were stimulated at 37 °C with 200 ng/ml of PMA. The chemiluminescence was assayed at 37 °C using a luminometer (Auto Lumat LB953, EG&G Berthold). The superoxide-producing activity was too low to be reliably measured by the cytochrome c reduction method (29, 36); the activity was less than 1% of that in activated human neutrophils, which is consistent with the results obtained by Cheng et al. (48).

Estimation of the Protein Level of p22phox and Cytosolic Regulatory Proteins—Transfected CHO cells were lysed by sonication. The sonicate was centrifuged for 10 min at 10,000 × g, and the supernatant was further centrifuged for 1 h at 100,000 × g. The resultant pellet was used.
as the membrane fraction for detection of the p22phox protein. On the other hand, total cell lysates of CHO and COS-7 cells were used for estimation of expression of p47phox, p67phox, Noxo1, and Noxa1. Membrane fractions or total cell lysates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with anti-p22phox polyclonal antibodies (Santa Cruz Biotechnology), anti-integrin β1 polyclonal antibodies (Santa Cruz Biotechnology), an anti-HA monoclonal antibody (Covance Research Products), or an anti-Myc monoclonal antibody (Roche Applied Science). The blots were developed using ECL-plus (Amersham Biosciences) for visualization of the antibodies, as described previously (46).

**Immunoprecipitation of Nox3 and p22phox Expressed in CHO Cells**—For analysis of the interaction between Nox3 and p22phox, CHO cells were transfected with the plasmid pcDNA3.0 for expression of Nox3 as a C-terminally Myc-tagged protein and/or the plasmid pEF-BOS-p22phox. After culture for 36 h, the transfected cells were lysed on ice for 10 min in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 2 mM Na3VO4, 10 mM NaF, 10 mM Na2P2O7, 10 mM okadaic acid, and the following protease inhibitors: leupeptin (2 μg/ml), pepstatin (2 μg/ml), chymostatin (10 μg/ml), aprotinin (20 μg/ml), trypsin inhibitor (10 μg/ml), and 4 mM p-aminophenylmethanesulfonyl fluoride. The lysate was centrifuged for 10 min at 10,000 × g, and the supernatant was precleared with protein G-Sepharose, the precipitates were subjected to SDS-PAGE, followed by immunoblot analysis with the anti-Myc monoclonal antibody or the anti-p22phox polyclonal antibodies.

For estimation of the Nox3-p22phox interaction, CHO cells were cotransfected with pEF-BOS-Nox3A1, pEF-BOS-p22phox, pEF-BOS-Myc-Noxa1, and pcDNA3.0-Nox3. The transfected cells were lysed as described above. Proteins were precipitated with preimmune sera or anti-p22phox sera, which were raised against the C-terminal 20 amino acids of human p22phox. The precipitates were analyzed by immunoblot with the anti-HA monoclonal antibody or the anti-p22phox polyclonal antibodies.

**Expression of mRNA for Noxo1 and Noxa1 in Human Fetal Kidneys**—To study the superoxide-producing activity of Nox3, we used COS-7 and HEK293 cells in which the activity of gp91phox/Nox2 and Nox1 has been successfully reconstituted (44–47). Transfection of these cells with an expression vector encoding the Nox3 cDNA alone leads to spontaneous production of a substantial amount of superoxide (Fig. 2, A and B). Here it should be noted that the Nox3-dependent superoxide production was observed without coexpression of an organizer (p47phox or Nox1) or an activator (p67phox or Nox1). This is in sharp contrast with that the superoxide production by gp91phox and Nox1 in these cells absolutely requires coexpression of both an organizer and an activator (44–47). The amount of superoxide produced by cells expressing Nox3 without an organizer or an activator was similar to that by cells containing gp91phox together with p47phox and p67phox in response to PMA (data not shown).

The Nox3-dependent superoxide production was abrogated by treatment of cells with diphenyleneiodonium chloride (Fig. 2, A and B), an inhibitor of the Nox family oxidases (44, 46). Furthermore, a mutant Nox3 protein carrying the substitution of His for Pro-413 in the NADPH-binding domain (Fig. 1A) failed to produce superoxide (Fig. 2, A and B). This substitution is equivalent to the Pro415H substitution in gp91phox, a mutation found in a CGD patient (54), which does not affect the protein integrity but results in a complete loss of the superoxide-producing activity (55). These findings further support the idea that Nox3 by itself catalyzes the superoxide production. Nox3 appears to be constitutively active, because the Nox3-dependent spontaneous production of superoxide occurred in the absence of PMA, a stimulant for the activation of gp91phox (29).

**Requirement of p22phox for the Nox3-dependent Superoxide Production**—The reconstitution of the superoxide-producing activity of gp91phox absolutely requires p22phox as the tightly associated partner (5–7). Transfection with the p22phox cDNA, however, is dispensable to the reconstitution when COS-7 and HEK293 cells are used; these cells express enough endogenous mRNAs for p22phox (44–47). In the reconstitution in CHO cells, on the other hand, the activity of gp91phox is totally dependent on the ectopic expression of p22phox, because CHO cells scarcely contain endogenous messages for p22phox (46). Hence we used CHO cells to investigate the role of p22phox in the Nox3-dependent superoxide production. As shown in Fig. 2C, the CHO cells ectopically expressing both Nox3 and p22phox produced superoxide in a manner independent of PMA; again, neither an organizer nor an activator was required. In contrast, the superoxide production was not observed in the CHO cells expressing Nox3 in the absence of p22phox. Similarly, expression of p22phox alone did not lead to superoxide production (Fig. 2D). These findings indicate that Nox3 forms a functional complex with p22phox to produce superoxide.

To investigate whether Nox3 physically interacts with p22phox, we expressed both p22phox and C-terminally Myc-tagged Nox3 in CHO cells and tested the possibility of the complex formation. As shown in Fig. 2E, Nox3 and p22phox were coimmunoprecipitated with the anti-Myc antibody. On the other hand, when a control antibody was used, neither Nox3 nor p22phox was precipitated (Fig. 2F). These findings indicate that Nox3 forms a heteromeric complex with p22phox. It is known that gp91phox stabilizes p22phox by forming a dimeric complex (5–7), and thus it is expected that Nox3 also increases the stability of p22phox. In CHO cells transfected with the p22phox cDNA alone, only a small amount of the protein p22phox was detected (Fig. 2F). Cotransfection with the Nox3 cDNA, on
in human fetal kidney (data not shown) where Nox3 is abundant (49, 50). As shown in Fig. 3, A–C, coexpression of both the classical organizer p47\textsubscript{phox} and the classical activator p67\textsubscript{phox} enhanced the Nox3-dependent superoxide production in COS-7, HEK293, and CHO cells. An approximate few-fold increase in the Nox3 activity was observed in the absence of PMA, and the addition of PMA resulted in a further enhancement. Intriguingly, coexpression of either p47\textsubscript{phox} or p67\textsubscript{phox} also enhanced the Nox3-dependent superoxide production; the effects of p47\textsubscript{phox} and p67\textsubscript{phox} seem to be additive (Fig. 3C). The p47\textsubscript{phox}-induced enhancement required treatment of cells with PMA, a PKC activator (Fig. 3D). The effect of PMA was completely reversed by GF109203X, a potent and selective inhibitor of PKC (31) (Fig. 3D), whereas the inhibitor did not affect the constitutive activity of Nox3 (data not shown). On the other hand, the p67\textsubscript{phox}-mediated enhancement was independent of PMA stimulation (Fig. 3C). Similar their own enhancing effects of p47\textsubscript{phox} and p67\textsubscript{phox} on the Nox3 activity were observed, when COS-7 cells were used instead of CHO cells (data not shown).

Role of the Interaction between p47\textsubscript{phox} and p22\textsubscript{phox} in Regulation of the Nox3 Activity—The interaction between p47\textsubscript{phox} and p22\textsubscript{phox} is mediated via binding of the p47\textsubscript{phox} SH3 domains to the p22\textsubscript{phox} PRR, and plays a crucial role in activation of gp91\textsubscript{phox} (16–19) (Fig. 1B). To clarify the role of the interaction in Nox3 regulation, we expressed a binding-defective mutant p22\textsubscript{phox} carrying the P156Q substitution in the PRR (16, 17) (Fig. 4A). The mutant protein was as active as the wild-type p22\textsubscript{phox} in the constitutive superoxide production by Nox3 (Fig. 4A). In cells expressing the mutant p22\textsubscript{phox}, however, p47\textsubscript{phox} failed to enhance superoxide production in response to PMA (Fig. 4B). Similarly, the W193R substitution in the N-terminal SH3 domain of p47\textsubscript{phox}, leading to a defective interaction with p22\textsubscript{phox} (18, 19), resulted in a complete loss of the enhancement by PMA (Fig. 4C). Thus the interaction between p47\textsubscript{phox} and p22\textsubscript{phox} appears to be crucial for the PMA-dependent enhancement of the Nox3 activity, indicative of a link between p22\textsubscript{phox} and Nox3. On the other hand, the P156Q substitution in p22\textsubscript{phox} did not affect the p67\textsubscript{phox}-mediated enhancement of the Nox3 activity (Fig. 4D).

Effects of Amino Acid Substitutions of p67\textsubscript{phox} on Nox3 Regulation—In the activation of gp91\textsubscript{phox}, p67\textsubscript{phox} functions both by interacting with Rac via the N-terminal TPR domain and by using the activation domain C-terminal to the Rac-binding domain (Refs. 36–39; see Fig. 1B). The R102E substitution, leading to an impaired binding to Rac, and the V204A substitution in the activation domain result in a loss of the activity to support activation of gp91\textsubscript{phox} (36, 39). The same effects were observed in the reconstitution in CHO cells (Fig. 5A). In contrast, p67\textsubscript{phox} (R102E) was capable of enhancing the Nox3-dependent superoxide production to the same extent as the wild-type one (Fig. 5B). Similarly, p67\textsubscript{phox} (V204A) was also as active as the wild-type protein (Fig. 5B), which is consistent with a recent finding that p67\textsubscript{phox} (V204A) is effective in activating Nox3 (48).

 Regulation of the Nox3 Activity by Noxo1—We next investigated whether the Nox3 activity can be regulated by the novel organizer Noxo1 and the novel activator Noxa1, the combination of which is suitable for activation of Nox1 (44–47). Coexpression of Noxo1 in COS-7 cells (Fig. 6A) or CHO cells (data not shown) enhanced the constitutive Nox3 activity; the enhancement was independent of PMA. We have previously shown that Noxo1, as well as p47\textsubscript{phox}, interacts with p22\textsubscript{phox} in the yeast two-hybrid system, and the Noxo1 SH3 domains directly bind to the PRR of p22\textsubscript{phox} in vitro (Ref. 46; see Fig. 1C). When Noxo1 and p22\textsubscript{phox} were coexpressed in CHO cells, they
were transfected with pcDNA3.0-Nox3 (wt) or pcDNA3.0-Nox3 (P413H). A suspension (1.0 ml) of the transfected cells (1 × 10⁶ cells) were preincubated for 5 min at 37 °C, and the chemiluminescence change was continuously monitored at 37 °C with an enhanced luminal-based substrate, DIOGENES, in the presence or absence of 5 μM diphenyleneiodonium chloride (DPI). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. C. CHO cells were cotransfected simultaneously with pcDNA3.0-Nox3 and pEF-BOS-p22phox, or with pcDNA3.0-Nox3 alone. After preincubation of the suspension (1.0 ml) of the transfected cells (1 × 10⁶ cells) for 5 min, the chemiluminescence change was continuously monitored with DIOGENES, and superoxide dismutase (SOD, 50 μg/ml) was added where indicated. D. CHO cells were transfected simultaneously with a pair of pcDNA3.0-Nox3 (wt) and pEF-BOS-p22phox or a pair of pcDNA3.0-Nox3 (P413H) and pEF-BOS-p22phox. Superoxide production was determined by chemiluminescence change using DIOGENES in the presence or absence of 5 μM diphenyleneiodonium chloride. Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. E. Triton X-100-soluble extracts (2 mg of proteins) from HEK293 cells, transfected with pcDNA3.0-Nox3-Myc and pEF-BOS-p22phox, were equally divided into two tubes, each of which was subjected to immunoprecipitation (IP) using the anti-Myc monoclonal antibody and the anti-p22phox polyclonal antibodies. For details, see “Experimental Procedures.” These experiments have been repeated more than three times with similar results. F. Membrane fractions (15 μg of proteins) of CHO cells transfected with pcDNA3.0-Nox3 and pEF-BOS-p22phox or with pcDNA3.0 vector and pEF-BOS-p22phox were prepared by differential centrifugation and analyzed by immunoblot with anti-p22phox polyclonal antibodies or with the anti-integrin β1 (a membrane maker protein) polyclonal antibodies as a loading control. For details, see “Experimental Procedures.” These experiments have been repeated more than three times with similar results.

were precipitated together by anti-p22phox sera (Fig. 6B), suggesting that the Noxa1-p22phox interaction indeed occurs in mammalian cells. The P156Q substitution in p22phox, defective in binding to Nox1 (46), led to an impaired Noxo1-dependent enhancement of the Nox3 activity (Fig. 6, C and D). In addition, a mutant Noxa1 carrying the W197R substitution, equivalent to the W193R substitution in p47phox, interacted neither with p22phox (Fig. 6E) nor enhanced the superoxide production by Nox3 (Fig. 6D). These findings indicate that the interaction between Nox1 and p22phox is crucial for the Nox1-induced enhancement of the Nox3 activity. This interaction is considered to be constitutive (46), in contrast with that between p47phox and p22phox, which may explain the reason why the Noxo1-mediated enhancement does not require PMA.

Regulation of the Nox3 Activity by Noxa1—It is known that the Noxa1 mRNA is expressed in rat inner ears and embryonic kidneys, whereas the Nox1 mRNA exists in rat embryonic kidneys but not in inner ears (52). The present PCR analysis using the cDNA panel also suggested that Noxa1 as well as Noxo1 is expressed in human fetal kidneys (data not shown). Noxa1 by itself marginally affected the superoxide production by Nox3 (Fig. 6A), which is in contrast with the observation that p67phox is capable of enhancing the Nox3 activity (Fig. 3C). Coexpression of Noxa1, however, led to a decrease in the Noxo1-induced enhancement of the superoxide production; the Nox3 activity in the presence of Noxo1 and Noxa1 was the same as that without these regulators (Fig. 6A). As shown in Fig. 7A, Noxa1 inhibited the Noxo1-dependent superoxide production in a dose-dependent manner. The dose-dependent inhibition by Noxa1 was also observed when CHO cells were used instead of COS-7 cells (data not shown).

We next studied the mechanism underlying the inhibition by Noxa1. Although Noxa1 is known to act as an activator for gp91phox and Nox1 (45, 46), it has remained unclear how Noxa1 functions. Judging from the conservation of the domain architecture (44–46) (see Fig. 1C), it seemed likely that Noxa1, as well as p67phox, functions both by interacting with Rac and by using the activation domain. As expected, a mutant Noxa1 carrying the R103E substitution, defective in binding to Rac (46), failed to activate gp91phox (Fig. 7, B and C), indicating that the interaction between Noxa1 and Rac is essential for the Noxo1- and Noxa1-dependent activation of gp91phox. In addition, a mutant Noxa1 with the V205A substitution in the activation domain, equivalent to the V204A substitution in p67phox, was incapable of activating gp91phox (Fig. 7C), which agrees with a recent observation that Noxa1 (V205A) is inac-
In the presence of both Noxo1 and p67phox, we tested the effect of heterologous combinations of the organizers and activators on the superoxide production by Nox3. The p47phox of the organizers and activators on the superoxide production (44–48), we tested the effect of heterologous combinations of the organizers and activators on the superoxide production by Nox3. As shown in Fig. 8A, the coexpression of p67phox marginally affected the Nox3 activity, which agrees with the observation by Cheng et al. (48) but differs from the results in CHO cells (Fig. 8B) and COS-7 cells (data not shown). Thus the effect of p67phox seems to be dependent on cell type. Although Noxa1 inhibited the Noxo1-dependent superoxide production by Nox3 even in HEK293 cells (Fig. 8C), less inhibition was observed compared with that in COS-7 cells (Fig. 8A), suggesting some cell-type dependence.

**Roles of Rac in Regulation of the Nox3 Activity**—Rac is known to be essential for activation of gp91phox in leukocytes (5–7). Consistent with the previous observation using leukemic cells (56), expression of a dominant negative form of Rac1 (T17N) in CHO cells (Fig. 9A) or COS-7 cells (data not shown) decreased in the p47phox- and p67phox-dependent superoxide production by gp91phox, indicating the involvement of Rac in these types of cells. On the other hand, Rac1 (T17N) exhibited only a marginal effect on the Nox3-dependent superoxide production without the organizers or activators (data not shown). Essentially the same results were obtained when COS-7 cells were used instead of CHO cells (data not shown). These findings suggest that Rac does not participate in the Nox3 activity.

Despite the absolute requirement of GTP-bound Rac for gp91phox activation (5–7), ectopic expression of an active form of Rac in CHO cells (Fig. 9B) was nearly identical to that without the organizers and activators in CHO cells (Fig. 8B) and COS-7 cells (data not shown); the inhibition was dependent on the amount of the p67phox protein expressed (data not shown). Thus the Nox1-mediated enhancement of the Nox3 activity can be negatively regulated by the oxidase activators p67phox and Noxa1 in these types of cells (Figs. 6A and 8B).

In HEK293 cells, Nox3 has been shown to be nearly maximally activated by Noxa1, activity which is further increased but to an only slight extent by p67phox or Nox1 (48). We also tested the Nox1-dependent oxidase activity of Nox3 using HEK293 cells. As shown in Fig. 8C, the coexpression of p67phox and Noxa1 further facilitated the p47phox-induced enhancement of the Nox3 activity (Figs. 3C and 8A).
in these cells (46, 57, 58). On the other hand, Cheng et al. (48) have reported that the oxidase activity of gp91phox in HEK293 cells requires expression of a constitutively active form of Rac.

Similarly, in the reconstitution of gp91phox in HeLa cells expressing p47phox and p67phox, PMA-elicited superoxide production was largely dependent on the presence of a constitutively active form of Rac.

**FIG. 4. Role of the interaction between p47phox and p22phox in the Nox3-dependent superoxide production.** A, CHO cells were transfected with pcDNA3.0-NOX3 and simultaneously with pEF-BOS-p22phox wt, pEF-BOS-p22phox P156Q, or the pEF-BOS vector. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections (upper). Protein levels of p22phox in the transfected cells were estimated by immunoblot analysis with the anti-p22phox polyclonal antibodies (lower). B, CHO cells were cotransfected with pcDNA3.0-NOX3 and pEF-BOS-p47phox wt and simultaneously with pEF-BOS-p22phox wt, pEF-BOS-p22phox P156Q, or the pEF-BOS vector. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections (upper). Protein levels of HA-tagged p47phox in the transfected cells were estimated by immunoblot analysis with an anti-HA monoclonal antibody (lower). C, CHO cells were cotransfected with pcDNA3.0-NOX3 and pEF-BOS-p22phox wt and simultaneously with pEF-BOS-HA-p47phox wt or pEF-BOS-HA-p47phox P156Q. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections (upper). Protein levels of HA-tagged p47phox in the transfected cells were estimated by immunoblot analysis with an anti-HA monoclonal antibody (lower).

**FIG. 5. Effects of amino acid substitutions in p67phox on the Nox3-dependent superoxide production.** A, CHO cells were cotransfected with pcDNA3.0-gp91phox, pEF-BOS-p22phox, and pEF-BOS-HA-p47phox, and simultaneously with pEF-BOS-Myc-p67phox wt, pEF-BOS-Myc-p67phox R102E, or pEF-BOS-Myc-p67phox V204A. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections (upper). Protein levels of wild-type or mutant Myc-tagged p67phox in the transfected cells were estimated by immunoblot analysis with an anti-Myc monoclonal antibody (lower). B, CHO cells were cotransfected with pcDNA3.0-NOX3, pEF-BOS-p22phox wt, pEF-BOS-p47phox wt, and simultaneously with pEF-BOS-Myc-p67phox P156Q, pEF-BOS-Myc-p67phox R102E, or pEF-BOS-Myc-p67phox V204A. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections.
active Rac1 (Q61L) (Fig. 9E), suggesting that endogenous Rac is not activated in the preparation of HeLa cells. Even in these cells, the Nox3-dependent superoxide production did not require expression of Rac1 (Q61L) and was not enhanced by the active Rac1 (Fig. 9E). Taken together, Rac does not appear to be involved in the Nox3 activity.

**DISCUSSION**

In the present study, we show that Nox3 functions together with p22phox to produce superoxide. p22phox not only forms a complex with Nox3 (Fig. 2) but also plays an essential role in the Nox3 activity (Fig. 2). Nox3 appears to be constitutively active, because the ectopic expression of Nox3 leads to a spontaneous production of superoxide, which requires neither coexpression of oxidase organizers or activators, nor cell treatment with stimulants such as PMA in all the cell types tested (Fig. 2). The constitutive Nox3 activity is comparable with the superoxide-producing activity of PMA-stimulated cells ectopically expressing gp91phox together with p47phox and p67phox. The organizer and activator proteins, albeit dispensable, can regulate the Nox3 activity (Figs. 3–8). The regulated activity appears to be important in a physiological context, because organizers and activators exist in inner ears and fetal kidneys (Ref. 52 and shown in the present study), where Nox3 is abundantly expressed. The organizers p47phox and Noxo1 are capable of enhancing the Nox3-dependent superoxide production (Figs. 4 and 6). The p47phox-dependent enhancement is further facilitated by p67phox and Noxa1, whereas the Noxo1-dependent enhancement is attenuated by the activators (Figs. 6–8). Thus the activators p67phox and Noxa1 regulate Nox3 positively or negatively, depending on the organizer involved. On the other hand, the small GTPase Rac does not appear to be involved in the Nox3 activity (Fig. 9).
FIG. 7. Dose-dependent inhibition by Noxa1 of the Noxo-enhanced Nox3 activity and effects of amino acid substitutions in Noxa1. A, COS-7 cells were transfected simultaneously with pcDNA3.0-Nox3 (1 μg), pEF-BOS-HA-Noxa1 (1 μg), and the indicated amount of pEF-BOS-Myc-Noxa1. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections (upper). Protein levels of Myc-tagged Noxa1 (middle) or HA-tagged Noxa1 (lower) in the transfected cells were estimated by immunoblot analysis with the anti-Myc or anti-HA monoclonal antibody, respectively. B and C, CHO cells were cotransfected with pcDNA3.0-gp91phox, pEF-BOS-p22phox, and pEF-BOS-HA-Noxa1, and simultaneously with pEF-BOS-Myc-Noxa1 (wt), pEF-BOS-Myc-Noxa1 (R103E), or pEF-BOS-Myc-Noxa1 (V205A). In B, protein levels of Myc-tagged Noxa1 (upper) or HA-tagged Noxa1 (lower) in the transfected cells were estimated by immunoblot analysis with the anti-Myc or anti-HA monoclonal antibody, respectively. Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections (C). D, CHO cells were cotransfected with pcDNA3.0-Nox3, pEF-BOS-p22phox, and pEF-BOS-Noxa1, and simultaneously with pEF-BOS-Myc-Noxa1 (wt), pEF-BOS-Myc-Noxa1 (R103E), or pEF-BOS-Myc-Noxa1 (V205A). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. *, p < 0.01 versus Noxa1 (wt); **, p < 0.05 versus Noxa1 (wt).

The requirement of p22phox for the Nox3 activity is evident from the present observation that the Nox3-dependent superoxide production requires coexpression of p22phox in CHO cells, which scarcely express the p22phox mRNA (Fig. 2). In these cells, functional expression of gp91phox and Nox1 is also dependent on the presence of p22phox (46); the possibility for the association of Nox1 with p22phox is supported by a recent study showing that Nox1 as well as gp91phox seems to directly interact with p22phox (59). As shown in the present study, Nox3 also appears to physically associate with p22phox (Fig. 2). The association of gp91phox with p22phox is not only functionally important but also crucial for the stability of both proteins. In CGD phagocytes lacking gp91phox, the p22phox protein is absent despite the abundant expression of the p22phox mRNA and vice versa (5–7). A similar stabilization of p22phox is likely caused by Nox3, because expression of Nox3 increases in the amount of p22phox at the protein level (Fig. 2). Thus Nox3 appears to interact with p22phox both physically and functionally. This conclusion is further supported by the findings that both p47phox- and Noxo1-dependent regulations of Nox3 are totally dependent on their interaction with p22phox (Figs. 4 and 6), for which dependence could not occur if p22phox were not linked to Nox3.

It has recently been shown that in mice Nox3 is highly expressed in the inner ear and required for formation of otocnia, which is indispensable to the perception of balance and gravity (51). It is, however, presently unknown whether Nox3 also exists in human ears and participates in otocnia formation. Because p22phox-deficient CGD patients have no problems with perception of balance and gravity, the present finding of the p22phox requirement for the Nox3 activity may suggest that Nox3 is not essential for otocnia formation in humans. Alternatively, it seems possible that a heretofore unidentified homologue of p22phox might form a functional heteromer with Nox3 in human ears.

Nox3 is different from gp91phox and Nox1 in that its activity does not seem to require the organizers and activators; this independence is observed in various types of cells such as CHO, COS-7, and HER293 cells (Fig. 2). On the other hand, the activities of gp91phox and Nox1 expressed in these cells absolutely require coexpression of both an organizer and an activator, indicating that these cells express only a trace amount, if any, of the organizers and activators. Indeed, in estimation by the reverse transcription-PCR method using HEK293 cells, none or much lower amounts of the mRNAs for the organizers and activators could be detected compared with those of control cells such as phagocytes and colon epithelial cells. In addition, it seems unlikely that Nox3 is activated by unknown homologues of the oxidase organizers and activators in these cells, because no other closely related proteins are uncovered by an extensive search of the genomic and EST data bases.

The organizer- and activator-independent superoxide production by Nox3 neither requires cell stimulants such as PMA, a potent activator of gp91phox, nor is affected by the addition of PMA (Fig. 3). In addition, serum deprivation does not affect the superoxide-producing activity of Nox3 expressed in COS-7 and CHO cells.2 Thus Nox3 appears to be at least partially in a constitutively active state, although its full activity requires oxidase organizers and activators in these cells, because no other closely related proteins are uncovered by an extensive search of the genomic and EST data bases.

The organizer- and activator-independent superoxide production by Nox3 neither requires cell stimulants such as PMA, a potent activator of gp91phox, nor is affected by the addition of PMA (Fig. 3). In addition, serum deprivation does not affect the superoxide-producing activity of Nox3 expressed in COS-7 and CHO cells. Thus Nox3 appears to be at least partially in a constitutively active state, although its full activity requires oxidase organizers and activators. Such stimulus-independent superoxide production is not exceptional among Nox enzymes. Nox1 and gp91phox produce superoxide without cell stimulation in the presence of Noxo1 (instead of p47phox) and an activator (p67phox or Noxa1), although the superoxide production is fur-

2 N. Ueno and H. Sumimoto, unpublished observations.
Although the superoxide production by Nox3 in the absence of the regulatory proteins does not require cell stimulants, the p47phox-induced enhancement of the Nox3 activity is totally dependent on stimulation with PMA, a potent activator of protein kinase C. The effect of PMA is abolished by the protein kinase C inhibitor GF109203X, supporting the idea for the involvement of protein kinase C (Fig. 3). As shown in the present study, the p47phox-induced enhancement is mediated via its SH3-dependent interaction with p22phox (Fig. 4), which is known to be regulated by phosphorylation of p47phox. The SH3 domains of p47phox are normally masked but become accessible to p22phox by a conformational change that can be induced by protein kinases such as protein kinase C (29, 30). Taken together, p47phox likely enhances the Nox3 activity via a phosphorylation-triggered interaction with p22phox. On the other hand, although the Noxo1-dependent enhancement of the Nox3 activity also requires the interaction of Noxo1 with p22phox, it is independent of cell stimulation with PMA (Fig. 6). This is consistent with our previous observation that Noxo1 in a resting state is capable of binding to p22phox (46). Thus the organizer appears to determine the dependence of the Nox3 activity on cell stimulation.

One of the unexpected findings in the present study is that the activators Noxa1 and p67phox can act as negative regulators of Nox3 under certain conditions; they suppress the Noxo1-enhanced Nox3 activity (Figs. 6A and 8B). In contrast, Noxa1 and p67phox can act as activators in the p47phox-induced enhancement (Figs. 3C and 8A). Thus the organizer likely determines whether Noxa1 and p67phox serve as active or negative regulators for Nox3. It seems likely that the negative regulation depends on cell types to some extent. Compared with CHO and COS-7 cells, the Noxo1-dependent Nox3 activation in HEK293 cells is blocked more slightly by Noxa1 and marginally by p67phox (Fig. 8C). This seems to be at least partially consistent with the observation by Cheng et al. (48) that only a very small increase in the Noxo1-dependent Nox3 activity is seen when p67phox or Noxa1 is simultaneously expressed. In addition, the existence of cell-type dependent effects of organizers and activators may be also supported by the finding that the p47phox- and p67phox-dependent enhancement of the Nox3 activity in HEK293 cells is higher than that in COS-7 cells (Fig. 3). The cell-type dependence is possibly due to intracellular signaling differences between the CHO and HEK293 backgrounds. The mechanism underlying the negative effect of the activators on the Noxo1-dependent Nox3 activity remains unknown at present. It may include the interaction of Noxa1 and p67phox with Nox1. A mutant p67phox protein carrying the W494R substitution in the C-terminal SH3 domain and a mutant Noxa1 with the corresponding substitution W436R in the N-terminal TPR domain are known to be regulated by phosphorylation of p47phox. Further studies are required for understanding the mechanism.

The small GTPase Rac is known to play an essential role in activation of gp91phox. Rac is recruited upon phagocyte stimulation to the membrane independently of p47phox or p67phox. At the membrane Rac is converted to the GTP-bound form, and GTP-Rac directly interacts with the N-terminal TPR domain of p67phox, an interaction which is crucial for the gp91phox activation (36). It is currently understood that Rac induces a conformational change of p67phox to the active state and/or assists the
FIG. 9. Role of Rac in the Nox3-dependent superoxide production. A, CHO cells were cotransfected with pcDNA3.0-gp91phox, pEF-BOS-p22phox, pEF-BOS-p47phox, and pEF-BOS-p67phox and simultaneously with or without Rac1 (T17N). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. B, CHO cells were cotransfected with pcDNA3.0-Nox3, pEF-BOS-p22phox, pEF-BOS-p47phox, and pEF-BOS-p67phox and simultaneously with or without Rac1 (T17N). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. C, CHO cells were cotransfected with pcDNA3.0-gp91phox, pEF-BOS-p22phox, pEF-BOS-Nox1, and pEF-BOS-Noxa1 and simultaneously with or without Rac1 (T17N). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. D, CHO cells were cotransfected with pcDNA3.0-Nox3, pEF-BOS-p22phox, pEF-BOS-Nox1, and pEF-BOS-Noxa1 and simultaneously with or without Rac1 (T17N). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. E, HeLa cells were cotransfected with pcDNA3.0-gp91phox, pEF-BOS-p47phox, and pEF-BOS-p67phox and simultaneously with or without Rac1 (Q61L). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean ± S.D. of the peak chemiluminescence values obtained from three independent transfections. F, HeLa cells were cotransfected with pcDNA3.0-Nox3 and simultaneously with or without Rac1 (Q61L). Superoxide production was assayed by chemiluminescence using DIOGENES in the presence or absence of PMA (200 ng/ml). Each graph represents the mean of the peak chemiluminescence values obtained from three independent transfections.

targeting of p67phox to gp91phox in the phagocyte oxidase activation (10, 12, 60). This small GTPase also appears to play a role in activation of Nox1. The inhibition of the conversion of Rac to the GDP-bound state results in an impaired activation of Nox1 in guinea pig gastric mucosal cells, and the impairment is restored by expression of a constitutively active form of Rac (61). On the other hand, the present findings indicate that Rac does not participate in the Nox3 activity. Expression of a dominant negative form of Rac1 does not affect the superoxide-producing activity of Nox3, whereas it results in a drastic decrease in the gp91phox-dependent superoxide production (Fig. 9). In addition, expression of a constitutively active form of Rac is not required for the superoxide-producing activity of Nox3 in HeLa cells where the superoxide production by gp91phox is largely dependent on the presence of the active Rac (Fig. 9). The proposal that Rac is not involved in regulation of Nox3 may be further supported by the finding that the Nox3 activity does not require the activator proteins such as p67phox (Fig. 2); p67phox is indispensable for the function of Rac in the gp91phox activation. In addition, a mutant p67phox or Nox1 protein, each defective in binding to Rac because of a mutation in the TPR domain, fully supports the Nox3-dependent superoxide production but fails to activate gp91phox (Figs. 5 and 7).

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