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Arachidonic Acid Induces Direct Interaction of the p67^{phox}-Rac Complex with the Phagocyte Oxidase Nox2, Leading to Superoxide Production*

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Background: The mechanism whereby arachidonic acid (AA) activates the phagocyte oxidase Nox2 is not well understood, except for the AA-induced conformational change of p47^{phox}, a partner of the Nox2 activator p67^{phox}.
Results: AA also triggers Rac-GTP formation and Nox2 interaction with the p67^{phox}·Rac-GTP complex.
Conclusion: AA regulates Nox2 assembly at multiple steps.
Significance: p67^{phox}-Nox2 interaction is a novel regulatory step.

The phagocyte NADPH oxidase Nox2, heterodimerized with p22^{phox} in the membrane, is dormant in resting cells but becomes activated upon cell stimulation to produce superoxide, a precursor of microbicidal oxidants. Nox2 activation requires two switches to be turned on simultaneously: a conformational change of the cytosolic protein p47^{phox} and GDP/GTP exchange on the small GTPase Rac. These proteins, in an active form, bind to their respective targets, p22^{phox} and p67^{phox}, leading to productive oxidase assembly at the membrane. Although arachidonic acid (AA) efficiently activates Nox2 both *in vivo* and *in vitro*, the mechanism has not been fully understood, except that AA induces p47^{phox} conformational change. Here we show that AA elicits GDP-to-GTP exchange on Rac at the cellular level, consistent with its role as a potent Nox2 activator. However, even when constitutively active forms of p47^{phox} and Rac1 are both expressed in HeLa cells, superoxide production by Nox2 is scarcely induced in the absence of AA. These active proteins also fail to effectively activate Nox2 in a cell-free reconstituted system without AA. Without affecting Rac-GTP binding to p67^{phox}, AA induces the direct interaction of Rac-GTP-bound p67^{phox} with the C-terminal cytosolic region of Nox2. p67^{phox}-Rac-Nox2 assembly and superoxide production are both abrogated by alanine substitution for Tyr-198, Leu-199, and Val-204 in the p67^{phox} activation domain that localizes the C-terminal to the Rac-binding domain. Thus the "third" switch (AA-inducible interaction of p67^{phox}·Rac-GTP with Nox2) is required to be turned on at the same time for Nox2 activation.

The NADPH oxidase (Nox)[2] family enzymes deliberately produce reactive oxygen species and, therefore, contribute to a variety of functions, including host defense, signal transduction, and hormone synthesis (1–5). The Nox oxidases contain two distinct hemes in the N-terminal transmembrane region and FAD- and NADPH-binding sites in the C-terminal cytoplasmic domain and, thus form a complete electron-transporting apparatus from NADPH to O_2 via FAD and hemes in a single protein. Nox2 (also known as gp91^{phox}), the original member of the Nox family, is highly expressed in professional phagocytes such as neutrophils. The phagocyte oxidase Nox2 is completely inactive in resting cells but becomes activated during phagocytosis of invading microbes to produce superoxide, a precursor of microbicidal reactive oxygen species (1–5). The significance of this oxidase in host defense is evident because recurrent and life-threatening infections occur in patients with chronic granulomatous disease whose phagocytes fail to kill pathogenic microbes because of the genetic defect in the Nox2-based reactive oxygen species-producing system (6, 7).

Superoxide production by Nox2 is elicited not only during phagocytosis but also in response to soluble stimulants such as arachidonic acid (AA) and phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC (8–11). Upon phagocyte stimulation, AA is released from membrane phospholipids. AA release for Nox2 activation is considered to be catalyzed by phospholipase A_2 enzymes such as cPLA$_2$ (12–14) and peroxyredoxin 6 (15, 16). Nox2 is stably dimerized with the membrane-integrated protein p22^{phox}, and the heterodimer assembles into the active complex with the small GTPase Rac and the specialized Nox-activating proteins p47^{phox} and p67^{phox}, which are recruited from the cytosol to the membrane upon cell stimulation (1–5). The assembly of the Nox2-based oxidase requires two switches to be turned on simultaneously: a conformational change of p47^{phox} and GDP-to-GTP exchange on Rac. The stimulus-induced conformational change of p47^{phox}, comprising 390 amino acid residues, allows the bis-SH3 domain of this protein to interact with the C-terminal proline-rich region (PRR) of p22^{phox}, an interaction essential for Nox2 activation

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[2] The abbreviations used are: Nox, NADPH oxidase; AA, arachidonic acid; PMA, phorbol 12-myristate 13-acetate; PRR, proline-rich region; AIR, autoinhibitory region; TPR, tetratricopeptide repeat; PBD, p21 (Cdc42/Rac)-binding domain; CBB, Coomassie Brilliant Blue.

(17, 18). The bis-SH3 domain is normally masked via an intramolecular association with the autoinhibitory region (AIR) of amino acid residues 286–340, which locates immediately C-terminal to the second SH3 domain (19–22) (see Fig. 1*A*). The release of the inhibitory association as a switch can be induced by direct action of AA (22, 23) or by PKC-catalyzed phosphorylation of multiple serine residues in p47^{phox}-AIR (19, 22–24).

The PKC activator PMA also elicits GDP-to-GTP exchange on Rac in neutrophils, another event required for Nox2 activation (25), although it has remained unclear whether AA triggers the exchange as well. Rac-GTP, but not Rac-GDP, directly interacts with the N-terminal tetratricopeptide repeat (TPR) domain of p67^{phox} (26–28), whose interaction is required for Nox2 activation. C-terminal to the Rac-binding TPR domain, p67^{phox} (composed of 526 amino acid residues) harbors the activation domain (amino acid residues 190–210), which is followed by two SH3 domains and a PB1 domain that intervenes between them (Fig. 1*A*). The activation domain is crucial for Nox2 activation but does not seem to participate in p67^{phox} interaction with Rac-GTP (29–31). Little is known about the molecular mechanism by which Rac-GTP-bound p67^{phox} activates Nox2, although Rac-GTP is presumed to induce a conformational change of p67^{phox}, which might direct the activation domain toward Nox2 for superoxide production.

The Nox2-based oxidase can be activated in a cell-free system reconstituted with the Nox2/p22^{phox}-abundant phagocyte membrane, p47^{phox}, p67^{phox}, and Rac-GTP. The activation is elicited with an *in vitro* activator, commonly represented by an anionic amphiphile such as AA or SDS, but not with PMA (32–34). A target of the amphiphiles is p47^{phox}. AA and SDS are each capable of directly disrupting the AIR-mediated inhibitory association in p47^{phox} to render the bis-SH3 domain in a state accessible to p22^{phox} (22). On the other hand, it has remained unclear whether the conformational change of p47^{phox} is enough to activate Nox2 in the presence of Rac-GTP.

In this study, we show that AA and SDS are each able to trigger GDP-to-GTP exchange on Rac in intact cells. These anionic amphiphiles do not affect binding of Rac-GTP to p67^{phox}, but allow the p67^{phox}·Rac-GTP complex to interact directly with Nox2. This interaction and the subsequent superoxide production are impaired by alanine substitution for Tyr-198, Leu-199, and Val-204 in the activation domain of p67^{phox}. Combined with the previous finding that p47^{phox} is a target of amphiphiles (22, 23), the present findings indicate that AA induces the assembly of the productive Nox2 complex by functioning at multiple steps.

EXPERIMENTAL PROCEDURES

Chemicals—AA, oleic acid, stearic acid, and palmitic acid were purchased from Nacalai Tesque. PMA was purchased from Sigma-Aldrich, and GF109203X was obtained from Biomol Research Laboratories. Other chemicals used were of the highest purity commercially available.

Plasmid Construction—The DNA fragments encoding the following human proteins were prepared as described previously (22, 31, 35, 36): full-length p47^{phox} (amino acid residues 1–390), p47-(SH3)$_2$ (amino acid residues 151–286), full-length p67^{phox} (amino acid residues 1–526), p67^{phox}-(1–212), p67^{phox}-(1–212/R102E), Rac1 (Q61L), full-length Nox2 (amino acid residues 1–570), Nox2-C (amino acid residues 384–570), full-length p22^{phox} (amino acid residues 1–195), p22^{phox}-C (amino acid residues 132–195), p22^{phox}-C (P156Q), and the p21 (Cdc42/Rac)-binding domain (PBD) of the protein kinase Pak (amino acid residues 66–147). The cDNA fragment for p47^{phox}-ΔAIR, lacking the region of amino acid residues 286–340, was amplified by PCR using the cDNA for full-length p47^{phox} as a template. The cDNA for the chimeric protein p67^{phox}-Rac1 (Q61L) that comprises p67^{phox}-(1–212), the 30-serine-residue linker, and Rac1 with the Q61L/C189S substitution were generated by PCR. Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. The DNA fragments were ligated to the following expression vectors: pGEX-6P (GE Healthcare Biosciences) or pMAL (New England Biolabs) for expression of proteins fused to GST or maltose-binding protein in *Escherichia coli*, respectively; pcDNA3 (Invitrogen) for expression of Nox2 in CHO and HeLa cells; and pEF-BOS (37) for expression of HA-, FLAG-, or Myc-tagged proteins in CHO and HeLa cells. All constructs were sequenced for confirmation of their identities.

Activation of the Nox2-based Oxidase in the Whole-cell System—CHO cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) with the following plasmids: 0.5 μg of pEF-BOS-Myc-p67^{phox}; 0.5 μg of pEF-BOS-FLAG-p47^{phox} or pEF-BOS-FLAG-p47^{phox}-ΔAIR; 1.0 μg of pcDNA3-Nox2; and 0.1 μg of pEF-BOS-p22^{phox}. Because HeLa cells express endogenous p22^{phox} but require expression of Rac1 (Q61L) for PMA-induced Nox2 activation (35, 36), HeLa cells were transfected using Lipofectamine transfection reagent (Invitrogen) with the following plasmids: 0.1 μg of pEF-BOS-Myc-p67^{phox}, 0.1 μg of pEF-BOS-FLAG-p47^{phox} or pEF-BOS-FLAG-p47^{phox}-ΔAIR, 1.0 μg of pcDNA3-Nox2, and 1.5 μg of pEF-BOS-Myc-Rac1 (Q61L). The transfected cells were cultured for 24 h in Ham's F12 medium (CHO cells) or DMEM (HeLa cells) containing 10% fetal bovine serum. For serum starvation, cells were washed with PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 (pH 7.4)), and cultured for 12 h in Ham's F12 medium (CHO cells) or DMEM (HeLa cells) containing 0.1% serum. Cells were harvested by incubation for 2 min at 37 °C with trypsin/EDTA and washed with PBS. Human neutrophils were prepared from fresh venous blood of healthy volunteers by dextran sedimentation, hypotonic lysis, and Conray/Ficoll method as described previously (22, 25). In the preparation, more than 98% of the cells were neutrophils.

CHO cells, HeLa cells, or human neutrophils were suspended in Hepes-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 17 mM Hepes (pH 7.4)), and preincubated for 30 min at 37 °C. The superoxide-producing activity was determined by superoxide dismutase-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (Diogenes, National Diagnostics), as described previously (38). After the addition of the enhanced luminol-based substrate, cells were preincubated for 5 min at 37 °C and subsequently stimulated at the same tem-

perature with the indicated concentrations of PMA, AA, oleic acid, stearic acid, palmitic acid, or SDS. The chemiluminescence change was monitored at 37 °C using a luminometer (Auto Lumat LB953, EG&G Berthold).

For estimation of protein levels of FLAG-p47^{phox}, Myc-p67^{phox}, Myc-Rac1, and p22^{phox}, proteins in cell lysates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with an anti-FLAG monoclonal antibody (Sigma-Aldrich), an anti-Myc monoclonal antibody (Roche Applied Science), and an anti-p22^{phox} polyclonal antibody (Santa Cruz Biotechnology), respectively. The blots were developed using ECL Plus (GE Healthcare Biosciences) for visualization of the antibodies.

Preparation of Recombinant Proteins—GST- or maltose-binding protein-tagged proteins were expressed in *E. coli* BL21 (Stratagene) and purified by glutathione-Sepharose-4B (GE Healthcare Biosciences) or amylose resin (New England Biolabs), respectively, according to the protocols of the manufacturers. For purification of recombinant Rac1 (Q61L) (the GTP-bound, active form of Rac1 carrying the Q61L/C189S substitution), full-length p67^{phox}, p67^{phox}-(1–212), p67^{phox}–Rac1 (Q61L), and p67^{phox} (Y198A/L199A/V204A)-Rac1 (Q61L), GST-tagged proteins were applied to glutathione-Sepharose-4B beads, and bound proteins were eluted from the beads by cleavage with PreScission protease (GE Healthcare Biosciences) according to the protocol of the manufacturer. Proteins were analyzed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue (CBB).

Cell-free Activation of the Phagocyte Oxidase Nox2—The membrane fraction of human neutrophils was prepared as described previously (31, 35, 36). The membranes (3 μg/ml) were mixed with 100 nM wild-type or mutant p47^{phox}, 100 nM p67^{phox}, and 100 nM Rac1 (Q61L) in 100 mM potassium phosphate (pH 7.0), containing 75 μM cytochrome *c*, 15 μM FAD, 1.0 mM $MgCl_2$, 1.0 mM EGTA, and 1.0 mM NaN_3. After incubation for 2.5 min at 25 °C with the indicated concentration of AA or SDS, the reaction was initiated by addition of 1.0 mM NADPH. The NADPH-dependent superoxide-producing activity was measured by determining the rate of superoxide dismutase-inhibitable ferricytochrome *c* reduction at 550–540 nm using a Hitachi 557 dual wavelength spectrophotometer. The superoxide-producing activity was represented as moles of superoxide produced per second per mole of cytochrome b_{558} heme.

An in Vitro Binding Assay Using Purified Proteins—For *in vitro* pull-down assays for p47^{phox} binding to p22^{phox}, 20 μg of GST alone or GST-p47^{phox} and 30 μg of maltose-binding protein-p22^{phox}-C were incubated for 30 min at 4 °C in 300 μl of 100 mM potassium phosphate (pH 7.0). A slurry of glutathione-Sepharose 4B beads was subsequently added, followed by further incubation for 30 min at 4 °C. After washing three times with the buffer above containing 0.5% Triton X-100, the proteins were eluted from the beads with 10 mM glutathione in 200 mM NaCl and 200 mM Tris-HCl (pH 8.0), containing 0.1% Triton X-100. The eluate was subjected to SDS-PAGE, followed by staining with CBB.

In vitro binding of Rac to p67^{phox} was performed as described previously (31, 36). Briefly, 20 μg of GST alone or GST-p67^{phox}-(1–212) with or without the R102E substitution was incubated for 15 min at 4 °C with 30 μg of Rac1 (Q61L) in 400 μl of 100 mM KCl, 100 mM potassium phosphate (pH 7.0) containing 0.005% Triton X-100. For *in vitro* interaction of p67^{phox}-Rac (Q61L) to Nox2-C, 50 μg of p67^{phox}-Rac (Q61L) and 20 μg of GST-Nox2-C (384–570) were incubated in 200 μl of 100 mM KCl and 100 mM potassium phosphate (pH 7.0) containing 0.005% Triton X-100. A slurry of glutathione-Sepharose 4B beads was added to the incubation mixture, followed by further incubation for 30 min at 4 °C. After washing four times with the buffer above, proteins were eluted from the beads with 20 mM glutathione in 200 mM NaCl and 200 mM Tris-HCl (pH 8.0), containing 0.1% Triton X-100. The eluate was subjected to SDS-PAGE, followed by staining with CBB or by immunoblot analysis with an anti-Rac monoclonal antibody (BD Biosciences).

Estimation of Rac Activation in Intact Cells—Rac activation in intact cells was estimated as described previously (25, 39). Briefly, HeLa cells, CHO cells, or human neutrophils 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 $MgCl_2$, and 5 mM EGTA containing 1% (v/v) protease inhibitor mixture (Sigma-Aldrich)). The lysate was centrifuged for 20 s at 12,000 × *g*, and the supernatant was incubated for 15 min with GST-Pak-PBD. Proteins were precipitated with glutathione-Sepharose-4B (GE Healthcare Biosciences), and the precipitants were analyzed by immunoblotting with the anti-Rac antibody.

RESULTS

AA Activates Nox2 in a Whole-cell System—It is well known that neutrophils produce superoxide in response to AA (8, 9), an anionic amphiphile that is also capable of activating the phagocyte oxidase Nox2 *in vitro* (32–34). On the other hand, the PKC activator PMA elicits superoxide production by intact cells but is unable to activate the oxidase in a cell-free system. To know the mechanism for oxidase activation by AA at the cellular level, we reconstituted the Nox2-based oxidase in CHO cells by ectopically expressing Nox2, p22^{phox}, p47^{phox}, and p67^{phox} and tested the effect of AA on superoxide production by these cells. In the whole-cell system, Nox2 was rapidly activated to produce superoxide in response to AA (Fig. 1*B*) as well as PMA (Fig. 1*C*). SDS, another anionic amphiphile that can activate the phagocyte oxidase in a cell-free system (33, 34), also triggered superoxide production in Nox2-expressing CHO cells (Fig. 1*C*). Superoxide production induced by AA terminated earlier than that induced by other stimulants, which seems to result from the fact that exogenous AA is incorporated immediately into membrane phospholipids (40). It is known that PMA is not rapidly metabolized, and, therefore, its action is sustained, leading to prolonged activation of Nox2. By contrast, the endogenous PMA analog diacylglycerol acts transiently because of its fast metabolism (41). Nox2 has been shown to be activated by *cis*-unsaturated fatty acids but not by *trans*-unsaturated or saturated ones (8, 42). Indeed, oleic acid activated the Nox2-based oxidase reconstituted in CHO cells (Fig. 1*D*) and the neutrophil oxidase Nox2 (Fig. 1*E*), albeit to a lesser extent than that by AA. On the other hand, Nox2 activation did not occur upon neutrophil stimulation with stearic acid or palmitic acid (Fig. 1*E*). These saturated fatty acids also failed to activate

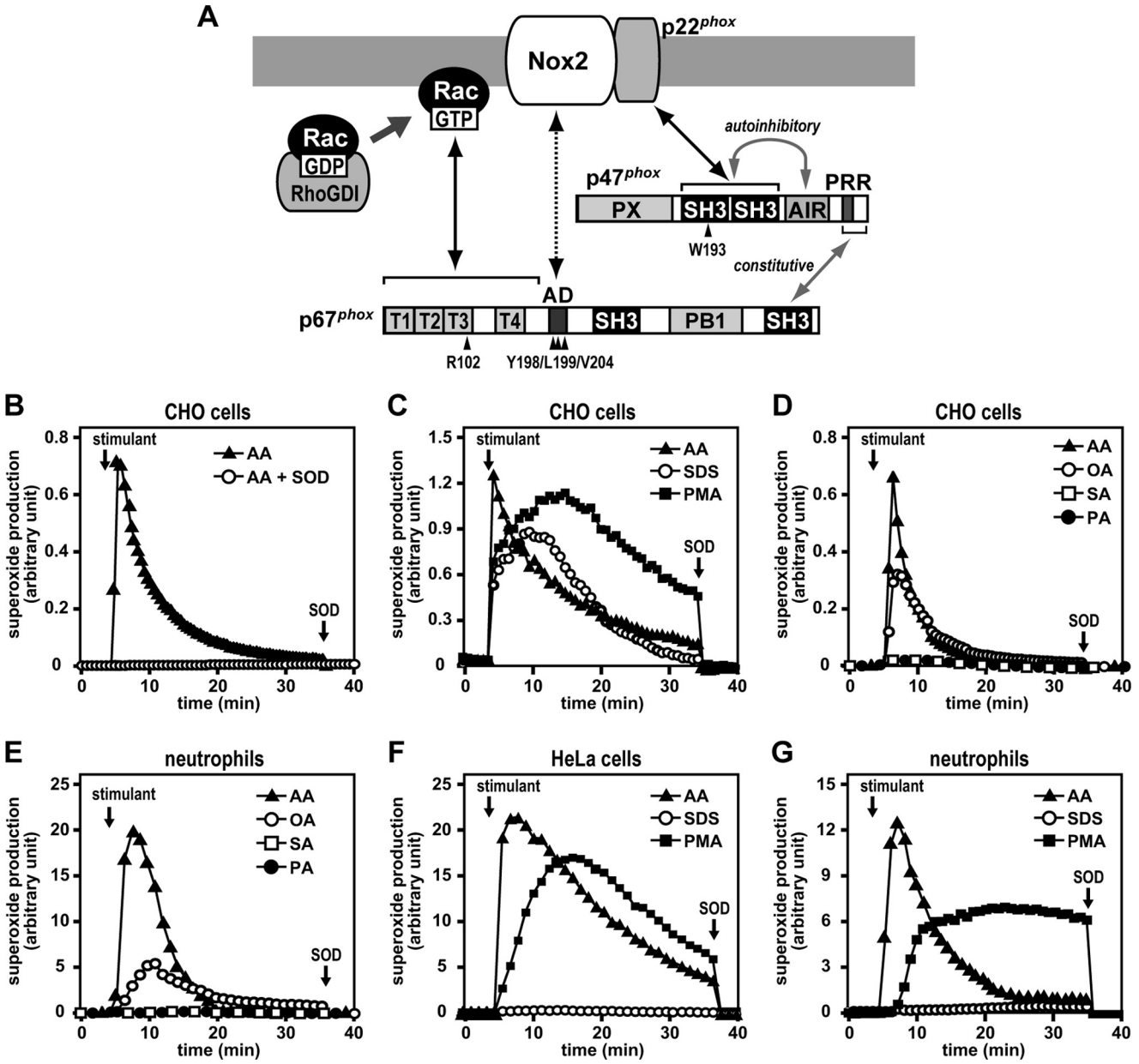

FIGURE 1. **AA- and SDS-induced superoxide production by the Nox2-based NADPH oxidase.** *A*, assembly of the Nox2-based NADPH oxidase. p47^{phox} comprises a phox-homology (*PX*) domain, two SH3 domains, an AIR, and a PRR. The cytosolic protein p47^{phox} constitutively associates with p67^{phox} via a tail-to-tail interaction between the p47^{phox} PRR and the p67^{phox} C-terminal SH3 domain. The bis-SH3 domain of p47^{phox} is normally masked with an AIR and becomes exposed upon cell stimulation to interact with the membrane protein p22^{phox}. The cytosolic oxidase-activating protein p67^{phox} harbors an N-terminal domain composed of four TPR motifs, followed by an activation domain (*AD*), two SH3 domains, and a PB1 domain. The TPR domain binds directly to Rac-GTP. Rac localizes to the cytosol in resting cells as a heterodimer with RhoGDI and translocates upon cell stimulation to the membrane to undergo GTP exchange for GDP. The activation domain of p67^{phox} is considered to interact directly with Nox2. Amino acid residues mutated in this study are indicated by *arrowheads*. *B–G*, superoxide production by the Nox2-based oxidase reconstituted in CHO or HeLa cells or by the neutrophil NADPH oxidase Nox2. CHO cells (*B–D*) or HeLa cells (*F*) were transfected simultaneously with pcDNA3-Nox2, pEF-BOS-p22^{phox}, pEF-BOS-FLAG-p47^{phox}, and pEF-BOS-Myc-p67^{phox} (*B–D*) or with pcDNA3-Nox2, pEF-BOS-FLAG-p47^{phox}, pEF-BOS-Myc-p67^{phox}, and pEF-BOS-Myc-Rac1 (Q61L) (*F*), as described under "Experimental Procedures." Transfected CHO cells were stimulated with AA (50 μM) in the presence or absence of superoxide dismutase (*B*); with AA (50 μM), SDS (100 μM), or PMA (200 ng/ml) (*C*); or with 20 μM AA, oleic acid (*OA*), stearic acid (*SA*), or palmitic acid (*PA*) (*D*). Human neutrophils were stimulated with 20 μM AA, oleic acid, stearic acid, or palmitic acid (*E*) or with AA (50 μM), SDS (100 μM), or PMA (200 ng/ml) (*G*). *F*, transfected HeLa cells were stimulated with AA (50 μM), SDS (100 μM), or PMA (200 ng/ml). The chemiluminescence change was monitored continuously with Diogenes. Superoxide dismutase (*SOD*, 50 μg/ml) was added where indicated. The experiments were repeated more than three times with similar results.

the Nox2-based oxidase reconstituted in CHO cells (Fig. 1*D*). We also used HeLa cells for analysis of *in vivo* activation of Nox2. As shown in Fig. 1*F*, the addition of AA to HeLa cells resulted in superoxide production to the same extent as that induced by stimulation with PMA. Thus AA effectively activates the Nox2-based oxidase in whole-cell reconstituted systems. On the other hand, SDS only marginally activated Nox2 in HeLa cells (Fig. 1*F*) as well as in neutrophils (Fig. 1*G*).

AA Induces GDP-to-GTP Exchange on Rac in Intact Cells—Superoxide production by Nox2 is triggered by turning on the following two switches simultaneously: p47^{phox} conformational change and GDP-to-GTP exchange on Rac (1–5). Although AA

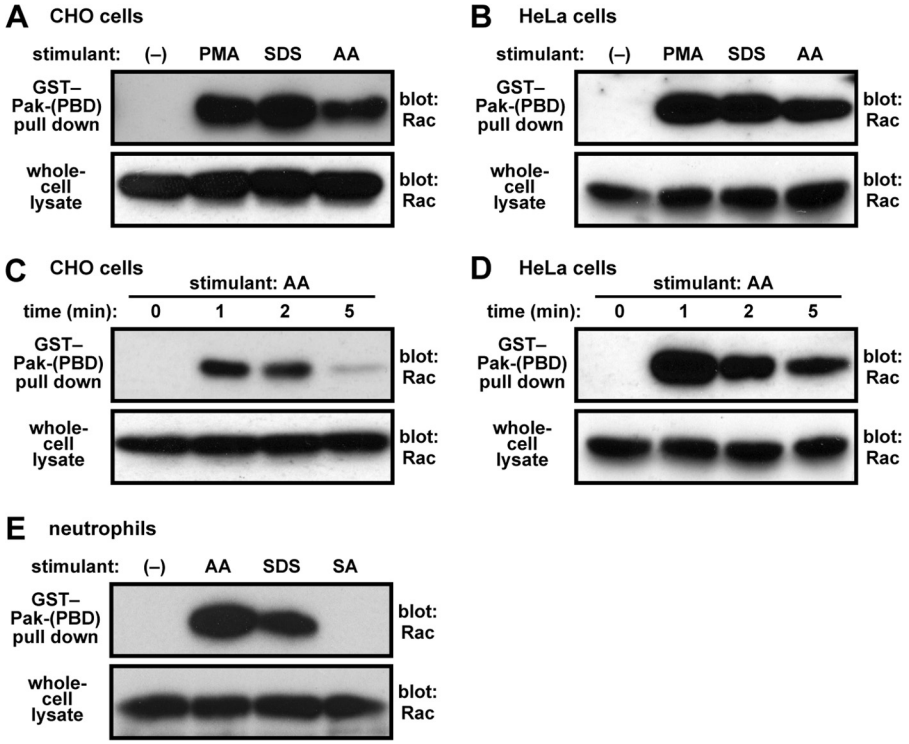

FIGURE 2. **Formation of GTP-bound Rac in cells stimulated with AA or SDS.** CHO cells (*A* and *C*), HeLa cells (*B* and *D*), or human neutrophils (*E*) were stimulated for 2 min (*A*, *B*, and *E*) or for the indicated times (*C* and *D*) with 50 μM AA, 100 μM SDS, 50 μM stearic acid (*SA*), or 200 ng/ml PMA, followed by cell lysis. Proteins in the lysate were mixed with GST-Pak-PBD and pulled down by glutathione-Sepharose-4B beads. Rac in the precipitant or the whole-cell lysate was estimated by immunoblot analysis with the anti-Rac antibody. The experiments were repeated more than three times with similar results.

is known to induce the conformational change of p47^{phox} (22, 23), the role for AA on Rac activation remains to be elucidated. To test the possibility that AA elicits conversion of Rac to the GTP-bound, active form at the cellular level, we performed a pull-down assay using GST-Pak-PBD (the Cdc42/Rac-binding domain of the protein kinase Pak), followed by immunoblot analysis with the anti-Rac antibody (25, 39). As we have shown previously in human neutrophils (25), PMA induced formation of Rac-GTP in CHO cells (Fig. 2*A*) and in HeLa cells (Fig. 2*B*). In both cell types, treatment with AA culminated in a rapid exchange of GTP for GDP on Rac (Fig. 2, *C* and *D*). In addition, AA also induced formation of Rac-GTP in human neutrophils, although the formation was not observed in response to the poor oxidase activator stearic acid (Fig. 2*E*). Furthermore, cell treatment with SDS led to the activation of Rac (Fig. 2, *A*, *B*, and *E*). These findings indicate that AA as well as SDS elicit GDP-to-GTP exchange on Rac, one of the two switches to be turned on for Nox2 activation.

Deletion of p47^{phox}-AIR Facilitates Nox2 Activation in a Whole-cell System—In the resting state, p47^{phox} is folded so that the bis-SH3 domain is inaccessible to its target p22^{phox}-PRR because of the intramolecular interaction with the AIR (19–22) (Fig. 1*A*). Indeed, a p47^{phox} protein lacking the AIR (p47^{phox}-ΔAIR) did interact with p22^{phox}-PRR under conditions where full-length p47^{phox} was incapable of binding to p22^{phox} (Fig. 3*A*), suggesting that p47^{phox}-ΔAIR likely serves as an active form. Expression of both the active form of p47^{phox} and the constitutively active, GTP-bound Rac1 (Q61L) is expected to induce Nox2-catalyzed superoxide production even without cell stimulants such as PMA and AA, given that turning on the two switches (the conformational change of p47^{phox} and formation of GTP-liganded Rac) is sufficient to activate the Nox2-based oxidase. Contrary to expectations, simultaneous expression of p47^{phox}-ΔAIR and Rac1 (Q61L) in HeLa cells did not result in superoxide production by Nox2 (Fig. 3*B*). The finding suggests that turning on the two switches of p47^{phox} and Rac is not sufficient to activate the Nox2-based oxidase *in vivo*. On the other hand, when HeLa cells were stimulated with SDS, p47^{phox}-ΔAIR was much more effective in activating Nox2 than wild-type p47^{phox} (Fig. 3, *B* and *C*), supporting the idea that p47^{phox}-ΔAIR functions as an active form. The finding also suggests that SDS functions as a poor activator of p47^{phox}. On the other hand, when Nox2 was reconstituted with wild-type p47^{phox} in CHO cells, SDS induced superoxide production to almost the same extent as AA and PMA (Fig. 1*C*), which implies the possibility that p47^{phox} may be more easily activated in CHO cells (see "Discussion"). In addition, although GF109203X, a potent inhibitor of PKC, blocked PMA-induced superoxide production, the inhibitor did not affect Nox2 activation elicited by AA, oleic acid, or SDS (Fig. 3, *C* and *D*), suggesting that these anionic amphiphiles function independently of PKC, possibly in a more direct manner.

Deletion of p47^{phox}-AIR Facilitates Nox2 Activation in a Cell-free System—We next studied the effect of the active form of p47^{phox} in a cell-free system for activation of the phagocyte NADPH oxidase. The system was reconstituted with the recombinant proteins p47^{phox}, p67^{phox}, and Rac1 (Q61L) (Fig. 4*A*) and human neutrophil membranes, in which the Nox2-p22^{phox} heterodimer is highly enriched (35, 36). As shown in Fig. 4*B*, in the presence of full-length p47^{phox}, the addition of

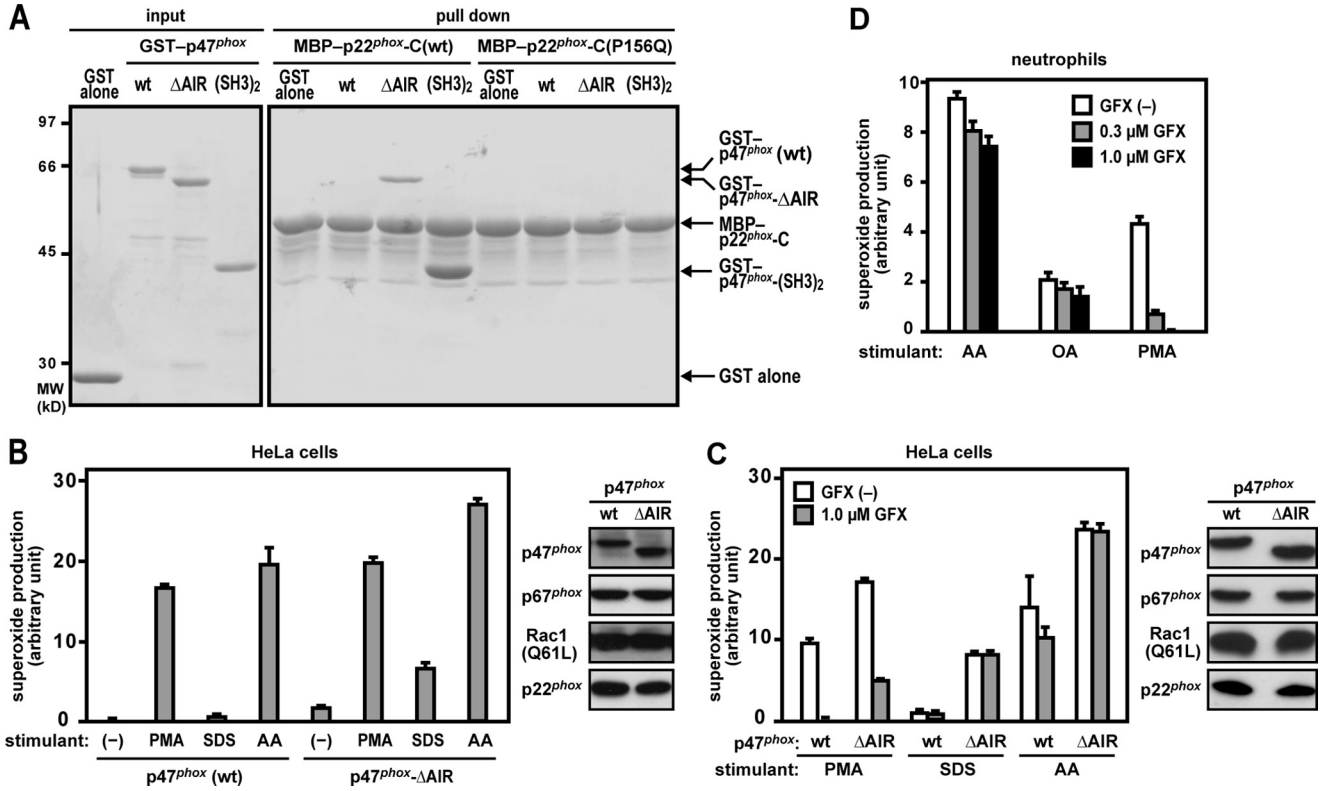

FIGURE 3. **Role for the p47^{phox} AIR in interaction with p22^{phox} and Nox2 activation.** *A*, effect of p47^{phox}-AIR truncation on binding to p22^{phox}. GST-p47^{phox} (WT), GST-p47^{phox}-ΔAIR, GST-p47^{phox}-(SH3)$_2$ (the bis-SH3 domain, amino acid residues 154–292), or GST alone was incubated with GST-p22^{phox}-C (WT) or GST-p22^{phox}-C (P156Q) and pulled down with amylose resin. The precipitated proteins were applied to SDS-PAGE, followed by staining with CBB, as described under "Experimental Procedures." Positions for marker proteins are indicated in kilodaltons. The data are representative of results from four independent experiments. *B–D*, superoxide production by the Nox2-based oxidase reconstituted in HeLa cells or by Nox2 in human neutrophils. HeLa cells were transfected simultaneously with pcDNA3-Nox2, pEF-BOS-p22^{phox}, pEF-BOS-Myc-p67^{phox}, pEF-BOS-Myc-Rac1 (Q61L), and pEF-BOS-FLAG-p47^{phox} (WT) or pEF-BOS-FLAG-p47^{phox}-ΔAIR. After preincubation in the presence (*C* and *D*) or absence (*B–D*) of GF109203X (*GFX*), the cells were stimulated with AA (50 μM), SDS (100 μM), or PMA (200 ng/ml). The chemiluminescence change was monitored continuously with 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 p47^{phox}, p67^{phox}, Rac1, and p22^{phox} were analyzed by immunoblotting as described under "Experimental Procedures."

SDS led to superoxide production with maximal activity at a concentration of 100 μM. When p47^{phox}-ΔAIR was used instead of the full-length protein, Nox2 was activated at lower concentrations of SDS (Fig. 4*B*), indicating that p47^{phox}-ΔAIR also serves as an active form *in vitro*. In addition, p47^{phox}-ΔAIR supported AA-induced superoxide production at lower concentrations compared with full-length p47^{phox}. At 5 μM AA, the superoxide-producing activity by p47^{phox}-ΔAIR was several times higher than that of the full-length protein, although full-length p47^{phox} was more active than p47^{phox}-ΔAIR at 50 μM AA (Fig. 4*C*). The concentrations of SDS and AA for maximal activation of Nox2 in HeLa cells (Fig. 4, *D* and *E*) were higher than those in the cell-free system (Fig. 4, *B* and *C*). In both the whole-cell and cell-free systems, AA fully functioned at concentrations lower than its critical micellar concentration of 73 μM (43), indicating that Nox2 activation by AA is not due to a detergent effect. It should be noted that, even in the presence of both p47^{phox}-ΔAIR and Rac1 (Q61L), only a marginal superoxide-producing activity was detected without stimulation with AA or SDS (Fig. 4, *B* and *C*), suggesting that the two switches of p47^{phox} and Rac are insufficient to elicit Nox2 activation *in vitro*. Taken together with a similar insufficiency *in vivo* (Fig. 3), it seems likely that a third switch localizes downstream of the formation of activated p47^{phox} or GTP-bound Rac and is required to be turned on for activation of the Nox2-based phagocyte oxidase.

AA Does Not Affect Rac Binding to p67^{phox}—To explore the third switch for Nox2 activation, we focused on steps after formation of Rac-GTP. The GTP-bound, active Rac is known to directly interact with the N-terminal domain of p67^{phox}, comprising four TPR motifs (26–28, Fig. 1*A*). This interaction is essential for Nox2 activation (26, 44), although Rac-GTP binds to p67^{phox} with a low affinity. Using purified Rac1 (Q61L) and GST-p67^{phox}-(1–212) (Fig. 5*A*), we performed a GST pull-down assay followed by immunoblot analysis for detecting low-affinity proteins. As shown in Fig. 5*B*, Rac1 (Q61L) interacted with GST-p67^{phox}-(1–212) in the absence of an anionic amphiphile. This interaction was abrogated by substitution of Glu for Arg-102 in the Rac-binding TPR domain of p67^{phox}, a mutation that completely impairs activation of the Nox2-based oxidase (26, 44). The addition of AA did not affect the interaction between Rac1 (Q61L) and GST-p67^{phox}-(1–212) (Fig. 5*B*). Similarly, the interaction was not enhanced or disrupted by the addition of SDS (Fig. 5*C*). These findings indicate that binding of Rac-GTP to p67^{phox} does not function as a switch for activating Nox2, at least in response to anionic amphiphiles such as AA and SDS.

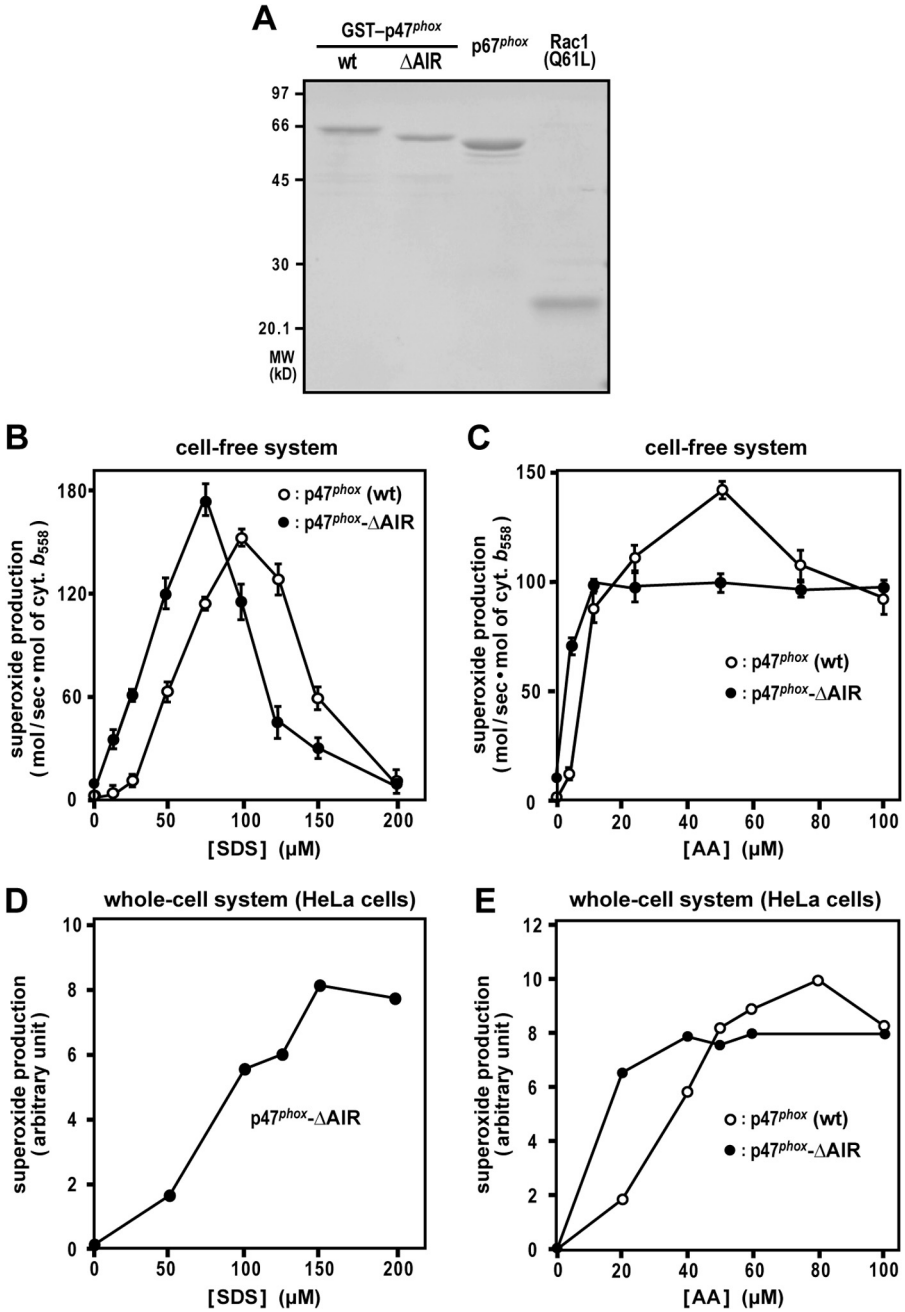

FIGURE 4. **Effect of p47^{phox}-AIR truncation on cell-free and whole-cell activation of the phagocyte oxidase Nox2.** *A*, SDS-PAGE analysis of proteins used in a cell-free activation system for the Nox2-based NADPH oxidase. Purified GST-p47^{phox} (WT), GST-p47^{phox}-ΔAIR, full-length p67^{phox}, and Rac1 (Q61L) were subjected to SDS-PAGE, followed by staining with CBB. Positions for marker proteins are indicated in kilodaltons. *B* and *C*, role for p47^{phox}-AIR in a cell-free activation system of the phagocyte oxidase Nox2. p67^{phox}, Rac1 (Q61L), and GST-p47^{phox} (WT) or GST-p47^{phox}-ΔAIR were used for a cell-free system activated with SDS (*B*) or AA (*C*) at the indicated concentrations, and the NADPH-dependent superoxide-producing activity was represented by the rate of superoxide dismutase-inhibitable cytochrome *c* reduction, as described under "Experimental Procedures." Data are mean ± S.D. from three independent experiments. *D* and *E*, HeLa cells were transfected simultaneously with pcDNA3-Nox2, pEF-BOS-p22^{phox}, pEF-BOS-Myc-p67^{phox}, pEF-BOS-Myc-Rac1 (Q61L), and pEF-BOS-FLAG-p47^{phox} (WT), or pEF-BOS-FLAG-p47^{phox}-ΔAIR. The cells were stimulated with the indicated concentration of SDS (*D*) or AA (*E*). The chemiluminescence change was monitored continuously with Diogenes. Each graph represents the mean of the chemiluminescence values integrated for 10 min, which were obtained from two independent transfections.

AA Induces Assembly of Nox2 with the p67^{phox}-Rac Complex—To investigate whether the p67^{phox}·Rac-GTP complex directly interacts with Nox2, we prepared a chimeric protein comprising the N-terminal region of p67^{phox} (amino acid residues 1–212) and Rac1 (Q61L) (Fig. 6*A*). The N-terminal region of p67^{phox} is known to be as effective as full-length p67^{phox} in activating the Nox2-based oxidase in the presence of Rac1-GTP and p47^{phox} in a cell-free system (22, 26). The p67^{phox}-Rac1 (Q61L) chimera is used due to the fact that p67^{phox} binds to Rac1 (Q61L) with a low affinity, and thus the chimeric protein can activate Nox2 at lower concentrations than those used when p67^{phox}-(1–212) and Rac1 (Q61L) were added separately (45). As shown in Fig. 6*B*, the present p67^{phox}-Rac1 (Q61L) chimeric protein supported superoxide produc-

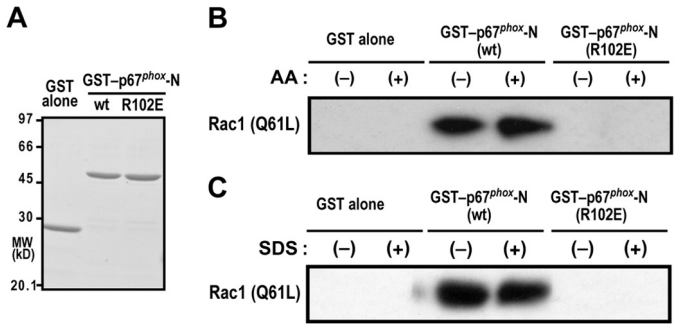

FIGURE 5. **Effect of AA and SDS on the interaction between p67^{phox} and Rac-GTP.** *A*, SDS-PAGE analysis of purified GST fusion proteins that were used in a GST pulldown assay for interaction with Rac. GST alone, GST-fused wild-type (WT) p67^{phox}-(1–212), and GST-p67^{phox}-(1–212/R102E), a mutant protein carrying the R102E substitution, were subjected to SDS-PAGE, followed by staining with CBB. Positions for marker proteins are indicated in kilodaltons. *MW*, molecular weight. *B* and *C*, effect of AA and SDS on the interaction of GST-p67^{phox} and Rac1 (Q61L). GST-p67^{phox}-(1–212) (WT) or GST-p67^{phox}-(1–212/R102E) was incubated with Rac1 (Q61L) in the presence or absence of 50 μM AA (*B*) or 100 μM SDS (*C*) and pulled down with glutathione-Sepharose-4B beads. The precipitated proteins were analyzed by immunoblotting with the anti-Rac antibody as described under "Experimental Procedures." Data are representative of results from four independent experiments.

tion by Nox2 in a cell-free system much more effectively than separated p67^{phox}-(1–212) and Rac1 (Q61L). A triple alanine substitution for Tyr-198, Leu-199, and Val-204 in the activation domain of the p67^{phox} moiety resulted in a complete loss of activation of the Nox2-based oxidase (Fig. 6*B*). This finding is consistent with the previous observation, using separated proteins, that these amino acid residues are not involved in binding to Rac-GTP but required for Nox2 activation both *in vivo* and *in vitro* (31).

We next examined whether the chimeric protein p67^{phox}-Rac1 (Q61L) is capable of directly interacting with Nox2 using purified proteins (Fig. 6*A*). Because Nox2 in the resting state is unable to bind to the substrate NAPDH and, therefore, Nox2 activation likely involves the increase in affinity for NADPH (1–5), we tested the NADPH-binding region that exists in the Nox2 C terminus (Nox2-C, amino acid residues 384–570) as a target of p67^{phox}-Rac1 (Q61L). In the absence of *in vitro* Nox2-activating reagents such as AA and SDS, the p67^{phox}-Rac1 (Q61L) chimera failed to associate with Nox2-C. In contrast, the addition of AA culminated in a direct interaction of p67^{phox}-Rac1 (Q61L) with Nox2-C (Fig. 6*C*). This interaction was completely impaired by the Y198A/L199A/V204A substitution in the p67^{phox} moiety, indicative of a crucial role for the activation domain. In addition, SDS also induced p67^{phox}-Rac1 (Q61L) binding to Nox2-C in a manner dependent on the activation domain of p67^{phox} (Fig. 6*D*). Therefore, p67^{phox}, in complex with Rac-GTP, appears to interact with Nox2 via the activation domain, whose interaction is induced by the Nox2-activating anionic amphiphiles AA and SDS.

DISCUSSION

The phagocyte oxidase Nox2, heterodimerized with p22^{phox}, becomes activated via its assembly with p47^{phox}, p67^{phox}, and Rac-GTP. The assembly requires two essential events: a conformational change of p47^{phox} for its binding to p22^{phox} and formation of GTP-liganded Rac for its association with p67^{phox} (1–5). AA, a potent activator of the phagocyte oxidase Nox2 both *in vivo* and *in vitro*, is known to induce one of the two events, *i.e.* the conformational change of p47^{phox} (22, 23). The change leads to the SH3-mediated binding of p47^{phox} to p22^{phox}, which is crucial for assembly of the Nox2-p22^{phox} heterodimer at the membrane with the cytosolic activating proteins p47^{phox} and p67^{phox}. p67^{phox} is recruited via a constitutive tail-to-tail interaction with p47^{phox} (1–5) (Fig. 1*A*). On the other hand, a more detailed molecular mechanism for AA-mediated Nox2 activation remains to be elucidated. Here we demonstrate that AA as well as SDS, another amphiphile activator of Nox2, elicits GTP exchange for GDP on Rac at the cellular level (Fig. 2).

In resting cells, Rac localizes to the cytosol as a heterodimer with Rho GDP dissociation inhibitor (RhoGDI), Rac being in the GDP-bound form. Upon cell stimulation, Rac translocates to the membrane in a manner independent of p47^{phox} and p67^{phox} (1–5). It has been reported that cell treatment with AA or SDS leads to membrane translocation of Rac (46–49) and that AA induces Rac-mediated processes in fibroblasts, although formation of Rac-GTP is not directly estimated (50). Rac activation involves dissociation of Rac-GDP from RhoGDI and GTP exchange for GDP on Rac, which is facilitated by guanine nucleotide exchange factors for Rac (51, 52). It is known that AA and SDS are each capable of dissociating Rac from RhoGDI *in vitro* (45, 46, 53). The dissociation of Rac and RhoGDI is known to promote Rac activation (51). As shown in the present study, AA by itself induces *in vivo* formation of GTP-liganded Rac (Fig. 2). This is consistent with the fact that the GTP-bound form of Rac is a prerequisite for Nox2 activation and that the activation can be triggered *in vivo* by the addition of AA (1–5). Cell treatment with another Nox2-activatable anionic amphiphile, SDS, also leads to GDP/GTP exchange on Rac (Fig. 2). It is known that PKC is known to phosphorylate RhoGDI to accelerate the dissociation of the Rac-RhoGDI complex (51, 52), and, indeed, the PKC activator PMA induces *in vivo* activation of Rac (Fig. 2) (24). Although AA is capable of activating PKC (54), it is unlikely that the amphiphile functions via PKC in Nox2 activation. This is because AA-induced superoxide production by Nox2 is not affected by GF109203X, a potent inhibitor of PKC (Fig. 3). In addition to AA-induced dissociation of Rac and RhoGDI, AA might further promote Rac activation by directly stimulating the function of Rac guanine nucleotide exchange factors such as Dock2, Vav1, and P-Rex, which are known to contribute to Nox2-catalyzed superoxide production in phagocytes (55–57). The possibility should be addressed in future studies.

In contrast to AA, SDS serves as a very poor stimulant for Nox2 activation in HeLa cells and neutrophils (Fig. 1), although SDS is able to fully induce formation of GTP-bound Rac in these cells (Fig. 2). The precise reason for the difference between SDS and AA at the cellular level remains unclear, but it has been reported that SDS has little effect on p47^{phox} in neutrophils and thus only marginally elicits superoxide production (58). Consistently, the potent Rac activator SDS (Fig. 2) is capable of substantially activating Nox2-based oxidase reconstituted with a constitutively active p47^{phox} (p47^{phox}-ΔAIR) but not with the wild-type protein in HeLa cells (Fig. 3). On the other hand, p47^{phox} may be easily activated in CHO cells

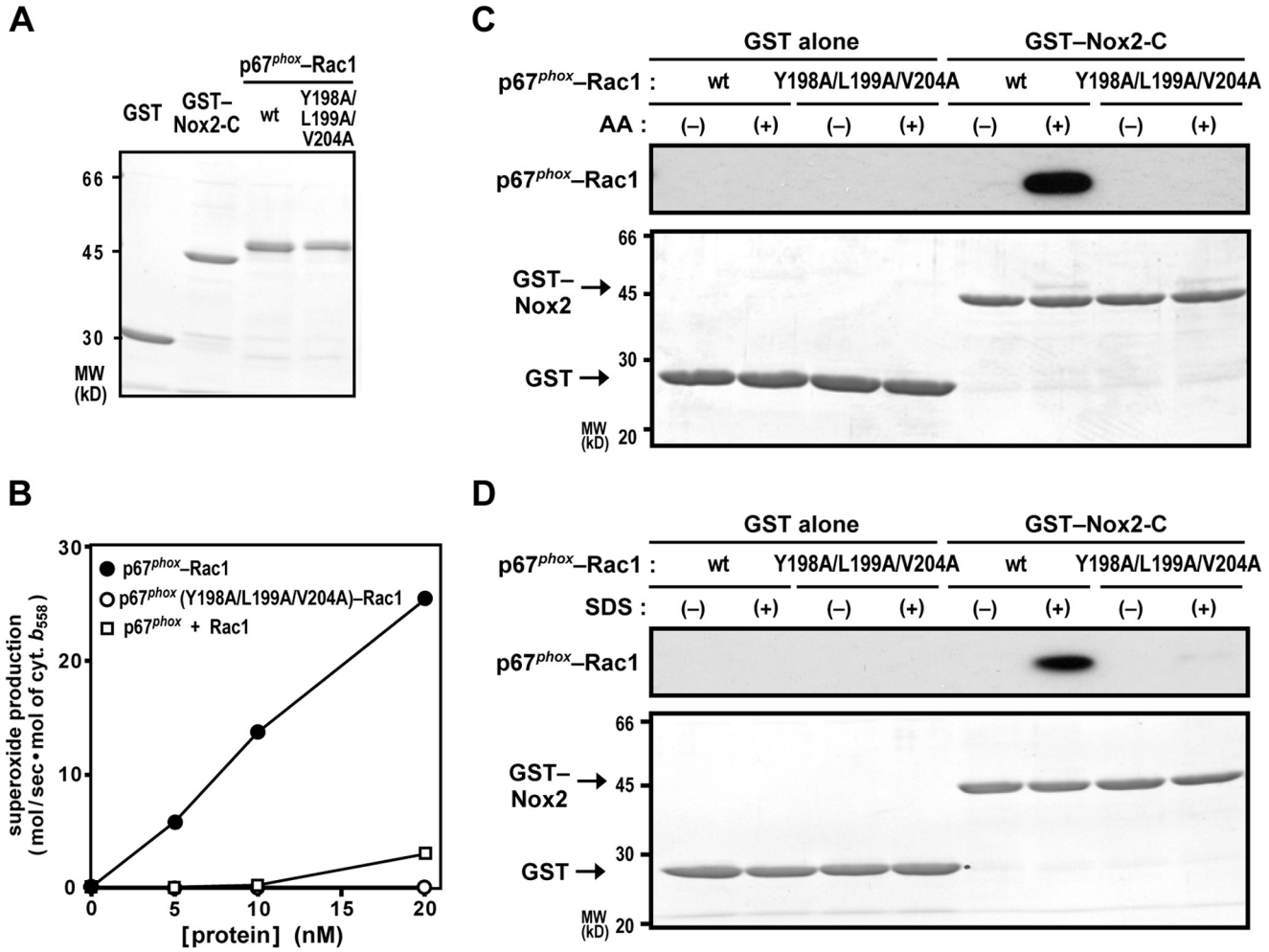

FIGURE 6. **Effect of AA and SDS on the interaction of the p67^{phox}·Rac-GTP complex with Nox2.** *A*, SDS-PAGE analysis of purified proteins used in this study. GST alone, GST-Nox2-C, the wild-type p67^{phox} (1–212)-Rac1 (Q61L) chimera, and a mutant p67^{phox}-Rac1 (Q61L) chimera carrying the Y198A/L199A/V204A substitution in the activation domain of the p67^{phox} moiety were subjected to SDS-PAGE, followed by staining with CBB. Positions for marker proteins are indicated in kilodaltons. *MW*, molecular weight. *B*, effect of the Y198A/L199A/V204A substitution in the activation domain of p67^{phox} on cell-free activation of the phagocyte oxidase Nox2. The wild-type p67^{phox}-Rac1 (Q61L), p67^{phox} (Y198A/L199A/V204A)-Rac1, or the isolated p67^{phox} (WT) and isolated Rac1 (Q61L) was used with p47^{phox} for a cell-free system activated with 50 μM AA, and the NADPH-dependent superoxide-producing activity was represented by the rate of superoxide dismutase-inhibitable cytochrome *c* reduction, as described under "Experimental Procedures." Data are mean from three independent experiments. *C* and *D*, effect of AA and SDS on the interaction of the p67^{phox}-Rac1 complex with Nox2-C. GST-Nox2-C or GST alone was incubated with the wild-type p67^{phox}-Rac1 or p67^{phox} (Y198A/L199A/V204A)-Rac1 in the presence or absence of 50 μM AA (*C*) or 100 μM SDS (*D*) and pulled down with glutathione-Sepharose-4B beads. The precipitated proteins were subjected to SDS-PAGE, followed by staining with CBB (*bottom panels*) and by immunoblot analysis with the anti-Rac antibody (*top panels*), as described under "Experimental Procedures." The data are representative of results from four independent experiments. Positions for marker proteins are indicated in kilodaltons.

(because of an unknown mechanism) because SDS is as active as AA and PMA in activation of the Nox2-based oxidase in CHO cells expressing wild-type p47^{phox} (Fig. 1).

This study also shows that deletion of the p47^{phox}-AIR, responsible for maintaining this protein in an inactive form in resting cells, facilitates superoxide production in cells stimulated with AA, SDS, and PMA (Figs. 3 and 4). Similarly, p47^{phox}-ΔAIR supports cell-free activation of Nox2 at lower concentrations of AA or SDS compared with wild-type p47^{phox} (Fig. 4). However, even when a constitutively active p47^{phox} (p47^{phox}-ΔAIR) and a constitutively active GTP-bound Rac1 (Q61L) are both present, Nox2 activation still requires a cell stimulant, such as AA or PMA, in intact cells (Fig. 3) or an anionic amphiphile, such as AA or SDS, in a cell-free system (Fig. 4). These findings indicate that a heretofore unidentified event serves as the third switch for Nox2 activation. It is well established that Rac-GTP, but not Rac-GDP, interacts with the N-terminal TPR domain of p67^{phox} (26, 27), which plays an essential role in Nox2 activation (26, 39). The interaction occurs in the absence of AA or SDS and is not increased by their presence (Fig. 5). Thus the amphiphile activators of Nox2 do not appear to regulate formation of the binary complex of p67^{phox} and Rac-GTP, an event that immediately follows GDP/GTP exchange on Rac.

The binary complex of p67^{phox} and Rac-GTP has been thought to induce a conformational change of Nox2, leading to superoxide production (59–62). It has been reported previously that Rac directly interacts with the Nox2-p22^{phox} heterodimer via the insert region, a surface-exposed α-helix that is unique to the Rho subfamily among Ras-related small GTPases (63). However, this region appears to be dispensable for Nox2 activation in both cell-free and whole-cell systems (35, 61, 62).

On the other hand, it has been considered possible that the activation domain of p67^{phox} participates in interaction with Nox2, although it has remained unclear whether the result of the conformational change in p67^{phox} is to augment the actual binding of p67^{phox} to gp91^{phox} or to endow p67^{phox} with an ability to elicit electron flow in gp91^{phox}. Here we demonstrate that AA and SDS each induce direct binding of Rac-complexed p67^{phox} to the NADPH-binding region of Nox2 (Nox2-C) as the third switch for Nox2 activation (Fig. 6) without affecting p67^{phox} interaction with Rac-GTP (Fig. 5). The role of the Nox2 NADPH-binding region as a target of p67^{phox}-Rac is consistent with a prevailing model hypothesizing that the increase in affinity for NADPH is crucial for Nox2 activation (1–5). Alanine substitution for Tyr-198, Leu-199, and Val-204 in the activation domain of p67^{phox} (amino acid residues 190–210) abrogates both the p67^{phox}-Rac interaction with Nox2 and subsequent Nox2 activation (Fig. 6), suggesting a crucial role for the activation domain. In this context, it should be noted that, although the N terminus of the activation domain is a part of an α-helix (amino acid residues 187–193) in a Rac-free p67^{phox} (28), this domain is flexible or disordered in p67^{phox} complexed with Rac-GTP (27). The Rac-induced flexibility may allow productive interaction of p67^{phox} with Nox2 in the presence of AA. It is also possible that AA may modulate Nox2 conformational states relevant to binding to the p67^{phox}-Rac complex because AA is known to be capable of directly inducing a conformational change of Nox2 (64). Thus the present study shows that AA directly triggers the three crucial events for Nox2 activation: conversion of p47^{phox} into the active conformation; GDP to GTP exchange on Rac; and interaction of the p67^{phox}·Rac-GTP complex with Nox2, *i.e.* the third switch identified here.

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