Arachidonic Acid and Phosphorylation Synergistically Induce a Conformational Change of p47<sub>phox</sub> to Activate the Phagocyte NADPH Oxidase*

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The superoxide-producing phagocyte NADPH oxidase can be activated by arachidonic acid (AA) or by phosphorylation of p47<sub>phox</sub> under cell-free conditions. The molecular mechanism underlying the activation, however, has remained largely unknown. Here we demonstrate that AA, at high concentrations (50–100 μM), induces direct interaction between the oxidase factors p47<sub>phox</sub> and p22<sub>phox</sub> in parallel with superoxide production. The interaction, being required for the oxidase activation, is mediated via the Src homology 3 (SH3) domains of p47<sub>phox</sub> (p47-(SH3)2), which are intramolecularly masked in a resting state. We also show that AA disrupts complexation of p47-(SH3)2 with its intramolecular target fragment (amino acids 286–340) without affecting association of p47-(SH3)2 with p22<sub>phox</sub>, indicating that the disruption plays a crucial role in the induced interaction with p22<sub>phox</sub>. Phosphorylation of p47<sub>phox</sub> by protein kinase C partially replaces the effects of AA; treatment of the SH3 target fragment with PKC in vitro results in a completely impaired interaction with p47-(SH3)2, and the same treatment of the full-length p47<sub>phox</sub> leads to both interaction with p22<sub>phox</sub> and oxidase activation without AA, but to a lesser extent. Furthermore, phosphorylated p47<sub>phox</sub> effectively binds to p22<sub>phox</sub> and activates the oxidase in the presence of AA at low concentrations (1–5 μM), where an unphosphorylated protein only slightly supports superoxide production. Thus AA, at high concentrations, fully induces the interaction of p47<sub>phox</sub> with p22<sub>phox</sub> by itself, whereas, at low concentrations, AA synergizes with phosphorylation of p47<sub>phox</sub> to facilitate the interaction, thereby activating the NADPH oxidase.

Professional phagocytes such as neutrophils and macrophages play crucial roles in host defense against microbial infection. One of the important mechanisms directed toward the killing of invading microorganisms is known to be the generation of microbicidal oxidants. Chronic granulomatous disease is an inherited disorder in which phagocytes fail to generate such oxidants, and thus patients lacking this antimicrobial pathway suffer from recurrent and life-threatening infections. The pathway necessarily involves the phagocyte NADPH oxidase, which is dormant in resting cells but becomes activated during phagocytosis to produce superoxide, a precursor of microbicidal oxidants (reviewed in Refs. 1–6). The activated oxidase is considered to be a complex containing the following proteins: a membrane-spanning flavocytochrome b<sub>558</sub>, comprising the two subunits gp91<sub>phox</sub> and p22<sub>phox</sub>, that serves as the catalytic core to transfer electrons from NADPH to molecular oxygen; and the three signaling proteins p47<sub>phox</sub>, p67<sub>phox</sub>, and the small GTPase Rac, that translocates from the cytoplasm to the membrane to assemble with the cytochrome upon cell stimulation. Chronic granulomatous disease is caused by a defect of any of the genes encoding these four polypeptides except Rac.

The phagocyte NADPH oxidase also can be activated in a cell-free system reconstituted with cytochrome b<sub>558</sub>, p47<sub>phox</sub>, p67<sub>phox</sub>, and the GTP-bound Rac. The in vitro activation requires anionic amphiphiles such as arachidonic acid (AA) and SDS (7–11). AA activates the NADPH oxidase not only in a cell-free system but also in intact phagocytic cells (12, 13). Superoxide production by whole cells in response to a variety of stimulants is blocked by inhibitors of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) that liberates AA from membrane phospholipids (14–16) and was abolished in cytosolic PLA<sub>2</sub>-deficient phagocytic cells (17). These observations suggest that AA acts as a second messenger for the oxidase activation in stimulated cells. In both in vivo and in vitro activations, however, the relatively high concentration of AA (50–100 μM) is required, which calls into question the physiological relevance of AA (18).

Although the mechanism whereby AA activates the oxidase is largely unknown, several lines of evidence suggest that AA interacts with p47<sub>phox</sub> (19–23) This protein harbors two Src homology 3 (SH3) domains, which specifically bind to a proline-rich region in the cytoplasmic tail of p22<sub>phox</sub> upon activation (19, 24). The induced p47<sub>phox</sub>-p22<sub>phox</sub> interaction likely plays a crucial role in activation of the NADPH oxidase; both the interaction and superoxide production are completely abrogated by a mutation of the invariant residue Trp-193 in the N-terminal SH3 domain of p47<sub>phox</sub> (25, 26) or by substitution of Gln for Pro-156 in the proline-rich region of p22<sub>phox</sub>, a mutation that occurs in a patient with chronic granulomatous disease (19, 24, 27). A resting form of p47<sub>phox</sub> is likely in a closed inactive conformation where the SH3 domains are masked via an intramolecular interaction with the C-terminal region of this protein (19, 22). Because the addition of AA to neutrophil

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cytosol leads to formation of a complex of p47\textsuperscript{phox} with p22\textsuperscript{phox} exogenously added (19), AA is considered to cause a conformational change of p47\textsuperscript{phox} to expose the SH3 domains. The possibility of p47\textsuperscript{phox} as a target of AA is also supported by a recent observation that a C-terminally truncated p47\textsuperscript{phox}, in which the intramolecular interaction does not occur because of a lack of the SH3 target region, is capable of both binding to p22\textsuperscript{phox} and activating the oxidase in the absence of AA (22). The precise mechanism for AA-induced conformational change of p47\textsuperscript{phox}, however, has remained to be elucidated.

Activation of the NADPH oxidase in stimulated neutrophils is also accompanied by phosphorylation of p47\textsuperscript{phox} at several serine residues in the C-terminal region (28). The temporal relationship between the phosphorylation of p47\textsuperscript{phox} and superoxide production suggests that the phosphorylation is involved in the mechanism of the oxidase activation (29–31). It has recently been demonstrated that phosphorylated p47\textsuperscript{phox} can support superoxide production in a cell-free system without AA (32, 33). The extent of the activation, however, is several-fold lower than that triggered by AA. It is also largely unknown about the mechanism underlying phosphorylation-induced activation of p47\textsuperscript{phox}.

As confirmed in the present study, AA at high concentrations (50–100 \(\mu\)M) disrupts the SH3-mediated intramolecular interaction in p47\textsuperscript{phox}, a disruption that appears to render this protein in a conformation capable of interacting with p22\textsuperscript{phox} and activating the NADPH oxidase (19, 22). Here we show that phosphorylation of p47\textsuperscript{phox} by PKC partially replaces the effects of AA; the phosphorylation results in a complete disruption of the intramolecular interaction with the C-terminal region, but leads to a weaker binding to p22\textsuperscript{phox} and a lesser activation of the oxidase. Intriguingly, the latter two activities induced by the phosphorylation are increased in the presence of AA at low concentrations (1–5 \(\mu\)M), where the lipid by itself only slightly activates p47\textsuperscript{phox}. Thus AA and phosphorylation synergistically act on p47\textsuperscript{phox} to promote the interaction with p22\textsuperscript{phox}, thereby activating the NADPH oxidase. In addition, we present evidence suggesting that the synergism functions in the oxidase activation in vivo.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The DNA fragments encoding the full-length of p47\textsuperscript{phox} (p47-F; amino acid residues 1–390), p47-(SH3)\textsubscript{2}-(151–286), p47-(3C-1–286), p47-(3C-1–340), p47-(3C-1–319), and p47-(286–340) were amplified from a cloned cDNA encoding human p47\textsuperscript{phox} by polymerase chain reaction using specific primers, as described previously (19, 34). The polymerase chain reaction products were also ligated to pPET-28a (Invitrogen) for expression of proteins fused to glutathione transferase (GST) or maltose-binding protein (MBP), respectively. The polymerase chain reaction using specific primers, as described previously (19, 34), was followed by ligation with or without AA or SDS for 2.5 min at room temperature in 100 mM potassium phosphate, pH 7.0, containing 75 \(\mu\)g of cytochrome c, 10 \(\mu\)M FAD, 1.0 mM EGTA, 1.0 mM MgCl\textsubscript{2}, and 1.0 mM NaN\textsubscript{3}. The reaction was initiated by the addition of NADPH (250 \(\mu\)M) to the reaction mixture. The NADPH-dependent superoxide-producing activity was measured by determining the rate of superoxide dismutase-inhibitable ferricytochrome c reduction at 550–540 nm with a dual wavelength spectrophotometer (Hitachi 557) (22, 25). The rates were calculated using an absorption coefficient of 19.1 nm\textsuperscript{-1} cm\textsuperscript{-1}.

**In Vitro Phosphorylation of Recombinant p47\textsuperscript{phox} by PKC**—Phosphorylation of recombinant GST-p47\textsuperscript{phox} was carried out using 40 \(\mu\)g of fusion protein in final volume of 200 \(\mu\)l. The reaction mixture contained 1 mM ATP, 1 mM dithiothreitol, 10 mM MgCl\textsubscript{2}, 0.5 mM CaCl\textsubscript{2}, 10 \(\mu\)g of phosphatidyserine, 10 ng of phorbol 12-myristate 13-acetate (PMA), and 5.3 units of PKC (human recombinant PKC/2, Calbiochem) in 20 mM Tris-HCl (pH 7.4). The lipids were added as mixed liposomes prepared by dissolving 10 mg/ml phosphatidylinerine in chloroform, removing the chloroform under a stream of nitrogen, and then sonating the dried lipids for 2 min in 0.2 ml of water. Incubation was carried out for 75 min at 37 \(^\circ\)C. Phosphorylation of p47\textsuperscript{phox} was confirmed by a retarded mobility of the protein on SDS-PAGE and by incorporation of the radioactivity from [\(\gamma\text{-}\text{32P}\)ATP according to the method of Park et al. (32).

**Superoxide Production by Intact Neutrophils**—Human neutrophils (1 \(\times\) 10\textsuperscript{6} cells) suspended in 1 ml of HEPES-buffered saline (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 2 mM glucose, 1 mM MgSO\textsubscript{4}, and 1 mM Ca\textsubscript{2+}) were preloaded with 100 \(\mu\)M GTP\textsubscript{S} followed by inhibitor 4-bromophenacyl bromide (BPB; Sigma) and incubated for 1 min in the absence or presence of PMA (100 ng/ml), thereby activating the oxidase in the absence of exogenous AA (19), which is considered to cause a conformational change of p47\textsuperscript{phox} and activating the NADPH oxidase (19, 22). The interaction is mediated by the SH3 domains of p47\textsuperscript{phox}, which is normally masked by a conformational interaction with the C-terminal region of p47\textsuperscript{phox} (19, 22). It is thus necessary that AA induces a conformational change of p47\textsuperscript{phox} to interact with p22\textsuperscript{phox} to investigate the nature of the induced interaction, we purified both His-tagged full-length p47\textsuperscript{phox} (His-p47-F; amino acids 1–390) and GST-p22-C and tested whether AA causes direct interaction between the purified proteins. In the absence of AA, His-p47-F was not precipitated with GST-p22-C coupled to glutathione-Sepharose 4B beads (Fig. 1A). The ad-
condition of AA to the mixture followed by washing culminated in coprecipitation of His-p47-F with GST-p22-C in a dose-dependent manner. Elimination of AA from the washing buffer abolished coprecipitated His-p47-F (data not shown), suggesting that AA reversibly induces the interaction between the proteins. The association appears to be specifically mediated via specific binding of the SH3 domains of p47phox to the proline-rich region of p22phox, because His-p47-F was not recovered when beads were coupled to GST alone (data not shown) or a mutant p22-C carrying the P156Q substitution (150 pmol) was incubated with 300 pmol of His-p47-F (amino acids 1–340) (A) or His-p47-(1–319) (B) in the presence of the indicated concentration of AA (A, C–E) or in the presence of 50 μM AA, palmitic acid (PA), or 8,11,14-eicosatrienoic acid (EET) (B). Proteins were pulled down with glutathione-Sepharose-4B and subjected to 10% SDS-PAGE, followed by staining with CBB. For details, see “Experimental Procedures.” The experiments have been repeated more than twice with similar results.

To test the effect of AA on the intramolecular interaction, we prepared the two tandem SH3 domains of p47phox (p47-(SH3)2; amino acids 151–286) and its target p47-(286–340) as GST and MBP fusion proteins, respectively. The addition of AA led to a disrupted interaction between p47-(SH3)2 and p47-(286–340) (Fig. 1E), a dependence that is essentially the same as that between p47-F and p22-C (Fig. 1A). In addition, in the absence of AA, p47-(1–340) was incapable of interacting with p22-C (Fig. 1C), whereas p47-(SH3)2-(1–286), lacking this region, did bind to p22-C (Fig. 1E). Thus the SH3-mediated intramolecular interaction seems to be fully maintained in p47-(1–340).

SDS also disrupted the p47-(SH3)2–p47-(286–340) interaction (data not shown), whereas palmitic acid did not (Fig. 2A).

**FIG. 2. Effect of AA on an intramolecular interaction in p47phox and on the SH3 domain-mediated interaction of p47phox with p22phox.** A, GST-p47-(SH3)2 (amino acids 151–286) (150 pmol) was incubated with MBP-p47-(286–340) (300 pmol) in the presence of the indicated concentration of AA or 50 μM palmitic acid (PA). B, GST-p47-(SH3)2 (150 pmol) was incubated with MBP-p22-C (300 pmol) in the presence of the indicated concentration of AA. C, GST-p47-(SH3)2 (150 pmol) was incubated with MBP-p47-(286–340) (300 pmol) and MBP-p22-C (1500 pmol) in the presence of the indicated concentration of AA. Proteins were pulled down with glutathione-Sepharose-4B and subjected to 10% SDS-PAGE followed by staining with CBB. For details, see “Experimental Procedures.” The experiments have been repeated more than twice with similar results.

AA Disrupts the SH3-mediated Intramolecular Interaction of p47phox—In a resting state, the SH3 domains of p47phox are considered to interact intramolecularly with the region C-terminal to the domains (amino acids 286–340), an interaction that likely prevents the domains from binding to p22phox (19, 22). The importance of the SH3 target region is confirmed by the observation that AA induced interaction between p47-(1–340) and p22-C in a concentration-dependent manner (Fig. 1C) that is identical to that between p47-F and p22-C (Fig. 1A). On the other hand, a p47phox, with a further C-terminal truncation, p47-(1–319) could bind to p22-C in the presence of a lower concentration of AA (Fig. 1D). In addition, in the absence of AA, p47-(1–340) was incapable of interacting with p22-C (Fig. 1C), whereas p47-(SH3)2-(1–286), lacking this region, did bind to p22-C (Fig. 1E). Thus the SH3-mediated intramolecular interaction seems to be fully maintained in p47-(1–340).

SDS also disrupted the p47-(SH3)2–p47-(286–340) interaction (data not shown), whereas palmitic acid did not (Fig. 2A).

**FIG. 1. AA-induced interaction of p47phox with p22phox.** GST-p22-C (amino acids 132–195) (150 pmol) or the one carrying the P156Q substitution (150 pmol) was incubated with 300 pmol of His-p47-F (amino acids 1–380) (A and B), His-p47-(1–340) (C), His-p47-(1–319) (D), or His-p47-(SH3)2 (150 pmol) in the presence of the indicated concentration of AA (A, C–E) or in the presence of 50 μM AA, palmitic acid (PA), or 8,11,14-eicosatrienoic acid (EET) (B). Proteins were pulled down with glutathione-Sepharose-4B and subjected to 10% SDS-PAGE, followed by staining with CBB. For details, see “Experimental Procedures.” The experiments have been repeated more than twice with similar results.
AA Does Not Affect the Interaction between p47-(SH3)2 and p22-C—We next tested effects of the amphiphiles on the interaction between p47-(SH3)2 and p22-C. The interaction is affected by neither AA (Fig. 2B) nor SDS (data not shown). This finding not only indicates that the amphiphile-induced disruption of the interaction with p47-(286–340) (Fig. 2A) is not because of a nonspecific effect of the amphiphiles on the p47phox SH3 domains but also suggests that the amphiphile-triggered binding of p47-F to p22-C (Fig. 1A) primarily results from disrupted intramolecular interaction, but not from enhancement of the interaction of the SH3 domains with p22-C. To confirm this possibility, we added AA to the mixture containing GST-p47-(SH3)2, MBP-p47-(286–340), and MBP-p22-C, and precipitated proteins with glutathione-Sepharose 4B beads followed by analysis of the precipitants by SDS-PAGE. Without AA, GST-p47-(SH3)2 was exclusively complexed with MBP-p47-(286–340) (Fig. 2C). With increasing concentrations of AA added, GST-p47-(SH3)2 was gradually associated with MBP-p22-C with concomitant disappearance of the complexed MBP-p47-(286–340) (Fig. 2C). In the presence of 100 μM AA, about 35% of added MBP-p22-C was pulled down under the conditions by densitometric analysis. Such a change of the interactions mediated by the p47phox SH3 domains was also observed, when SDS was used instead of AA (data not shown). Thus these experiments illustrate well the mechanism whereby AA (as well as SDS) activates p47phox, the amphiphile activators specifically disrupt the intramolecular interaction to allow the SH3 domains to associate with p22phox.

AA Enhances Interaction of p47-ΔC with p22-C—To know whether the disruption of the SH3-mediated interaction by itself fully induces association between p47phox and p22phox, we tested the p22phox binding activity of p47-ΔC-(1–286), a truncated protein that lacks the SH3 target region (amino acids 286–340) and is thus expected to harbor unmasked SH3 domains in a resting state. As shown in Fig. 1E, p47-ΔC weakly bound to p22-C in the absence of AA. Intriguingly, the binding of p47-ΔC was fully enhanced by AA at 25 μM, where neither induced binding of p47-F with p22-C nor disrupted interaction between p47-(SH3)2 and p47-(286–340) was observed (Fig. 1A or 2A, respectively). Densitometric analysis revealed that the amount of GST-p22-C binding to p47-ΔC in the absence of AA is about 9% of that in the presence of 25–100 μM AA. The findings suggest that AA acts on p47phox at at least two sites to fully induce the interaction with p22phox and that the disruption of the interaction between p47-(SH3)2 and p47-(286–340) serves as a limiting step in the induced binding to p22phox.

Phosphorylation of the SH3 Target Site of p47phox Results in Impaired Interaction with the SH3 Domains—It is well established that stimulation of human neutrophils leads to extensive phosphorylation of p47phox in parallel with superoxide production (28–31). The phosphorylated residues, nine to ten serines, are all located in the C-terminal quarter (amino acids 303–379) of p47phox (28). A recent study has shown that phosphorylated p47phox is active in a cell-free activation system of the NADPH oxidase (33), raising the possibility that phosphorylation induces activation of p47phox in a manner similar to that caused by AA. To address this question, we tested whether phosphorylation of p47phox affects the SH3-mediated intramolecular interaction or not. The intramolecular SH3 target region (amino acids 286–340) contains several serines that become phosphorylated by PKC in vitro (28) as well as in human neutrophils stimulated with PMA, a direct activator of PKC (37). Among them, Ser-303 or Ser-304 has been shown to be necessarily phosphorylated for the oxidase activation, because the double mutant p47phox S303A/S304A is much less active than the wild-type one when expressed in Epstein-Barr virus-transformed p47phox-deficient B cells (38).

We treated MDP-p47-(286–340), but not GST-p47-(SH3)2, with PKC in vitro confirmed that MDP-p47-(286–340) was phosphorylated, by incorporation of the radioactivity from [γ-32P]ATP (data not shown). As shown in Fig. 3A, the phosphorylated MDP-p47-(286–340) was incapable of interacting with GST-p47-(SH3)2, whereas an unphosphorylated one stably bound to the SH3 domains. In the mixture containing MDP-p47-(286–340) phosphorylated by PKC, MBP-p22-C, and GST-p47-(SH3)2, a complex of GST-p47-(SH3)2 with MBP-p22-C was detected but not with the phosphorylated protein (Fig. 3B). In contrast, unphosphorylated MDP-p47-(286–340) stably bound to GST-p47-(SH3)2, even in the presence of MBP-p22-C (Fig. 3B). Thus PKC-mediated phosphorylation of the intramolecular SH3 target site of p47phox results in impaired interaction with the SH3 domains, which in turn may render the domains accessible to p22phox.

PKC-mediated Phosphorylation of p47phox Renders This Pro-
tein in a Conformation Capable of Weakly Binding to p22phox.—To confirm whether phosphorylated p47phox indeed binds to p22phox, we treated His-p47-F with PKC in vitro and tested its ability to interact with p22phox. We confirmed that the protein was phosphorylated by a retarded mobility of the protein on SDS-PAGE (Fig. 3C) as well as by incorporation of the radioactivity from [γ-32P]ATP (Fig. 3D) in parallel experiments. Unexpectedly, we could not detect p47-F on SDS-PAGE analysis of proteins precipitated with GST-p22-C, followed by staining with CBB (data not shown). Immunoblot analysis with an anti-p47phox antibody, however, revealed that the phosphorylated p47-F interacts with p22-C (Fig. 3E). Without the treatment with PKC, His-p47-F hardly bound to p22-C (Fig. 3E). Thus PKC-mediated phosphorylation of p47phox causes a weak interaction with p22phox.

We also phosphorylated His-p47-(1–340) with PKC and tested its ability to bind to p22phox. Because the residues phosphorylated by PKC in vitro is exclusively present in the C-terminal quarter of p47phox (amino acids 303–379) (37), it is expected that p47-(1–340) becomes phosphorylated solely at residues within the intramolecular SH3 target region (amino acids 286–340). As shown in Fig. 3E, p22-C interacted with phosphorylated His-p47-(1–340) but not with an unphosphorylated protein. This observation further supports that PKC-mediated phosphorylation of the intramolecular SH3 target region plays a crucial role in a conformational change of p47phox.

The NADPH Oxidase Can be Activated without AA by Phosphorylated Wild-type p47phox, Not by the One with the S303A/S304A Substitution, under Cell-free Conditions—The results described above indicate that phosphorylation of p47phox completely replaces AA in disruption of the SH3-mediated intramolecular interaction but partially does in induction of the binding to p22phox. We next tested the effect of the PKC-mediated phosphorylation on activation of the phagocyte NADPH oxidase. In the absence of AA, the oxidase was activated by phosphorylated GST-p47-(1–340) (Fig. 4, A and B) or by phosphorylated GST-p47-F (Fig. 4C) in a cell-free system reconstituted with human neutrophil membranes that contain a high amount of the catalytic core cytochrome b556, GST-p67-N, and the small GTPase Rac2 preloaded with GTP-S. The activation required a longer lag period, and its extent was lower by several folds than that induced by AA (Fig. 4, A and B), which is consistent with a previous observation that phosphorylated p47phox is several fold less active than SDS in a cell-free system (33). On the other hand, unphosphorylated proteins were incapable of activating the NADPH oxidase in the AA-independent cell-free system (Fig. 4, B and C). These findings indicate that PKC-mediated phosphorylation of p47phox can partially replace AA in the oxidase activation as well.

To clarify the role for phosphorylation of the intramolecular SH3 target region (amino acids 286–340) in the oxidase activation, we prepared mutant proteins carrying substitution of the kinase-insensitive alanine for Ser-303/Ser-304 or Ser-328, respectively. The three serines are known to become phosphorylated by PKC in vitro (38) as well as by incorporation of [γ-32P]ATP (Fig. 3D) in parallel experiments. Unexpectedly, we could not detect p47-F on SDS-PAGE analysis of proteins precipitated with GST-p22-C, followed by staining with CBB (data not shown). Immunoblot analysis with an anti-p47phox antibody, however, revealed that the phosphorylated p47-F interacts with p22-C (Fig. 3E). Without the treatment with PKC, His-p47-F hardly bound to p22-C (Fig. 3E). Thus PKC-mediated phosphorylation of p47phox causes a weak interaction with p22phox.

We also phosphorylated His-p47-(1–340) with PKC and tested its ability to bind to p22phox. Because the residues phosphorylated by PKC in vitro is exclusively present in the C-terminal quarter of p47phox (amino acids 303–379) (37), it is expected that p47-(1–340) becomes phosphorylated solely at residues within the intramolecular SH3 target region (amino acids 286–340). As shown in Fig. 3E, p22-C interacted with phosphorylated His-p47-(1–340) but not with an unphosphorylated protein. This observation further supports that PKC-mediated phosphorylation of the intramolecular SH3 target region plays a crucial role in a conformational change of p47phox.

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To clarify the role for phosphorylation of the intramolecular SH3 target region (amino acids 286–340) in the oxidase activation, we prepared mutant proteins carrying substitution of the kinase-insensitive alanine for Ser-303/Ser-304 or Ser-328, namely GST-p47-(1–340), S303A/S304A or GST-p47-(1–340), S328A, respectively. The three serines are known to become phosphorylated in stimulated human neutrophils (28) as well as by PKC in vitro (37). Even when treated with PKC, p47-(1–340), S303A/S304A was incapable of activating the oxidase under the conditions without AA, although about a half of the activity was observed with phosphorylated p47-(1–340), S328A (Fig. 4D). The results are consistent with the observations that the S303A/S304A or S328A substitution of p47phox results in an almost complete or partial loss of the ability to support superoxide production, respectively, when expressed in Epstein-Barr virus-transformed p47phox-deficient B cells (38, 39). On the other hand, in the presence of AA, these mutant proteins are as active as the wild-type p47phox (Fig. 4E), excluding the possibility that the substitutions result in a loss of the potential to activate the oxidase. Thus PKC-mediated phosphorylation of the intramolecular SH3 target region likely participates in rendering p47phox in an active conformation.

AA and Phosphorylation of p47phox Synergistically Activate the NADPH Oxidase Under Cell-free Conditions—As described above, phosphorylation of p47phox evoked a complete disruption of the SH3-mediated intramolecular interaction with the C-terminal region (amino acids 286–340) (Fig. 3A) but induced only a weak binding to p22phox (Fig. 3E) that is also mediated via the SH3 domains. Consistent with this, the phosphorylation served as a poor activator of the oxidase in vitro (Fig. 4A). A longer lag time was observed in the activation by phosphorylated p47phox, compared with that by AA (Fig. 4A). Although...
the reason for the difference is not clear at present, it is possible that the weak binding of phosphorylated p47^phox to p22^phox is because of the low association rate, which likely leads to delayed activation of the oxidase. On the other hand, AA at lower concentrations could not disrupt the intramolecular interaction (Fig. 2A) but did enhance the binding of p47-DC, lacking the SH3 target, to p22^phox (Fig. 1E).

These findings raise the possibility that, in the presence of low concentrations of AA, phosphorylated p47^phox effectively binds to p22^phox to activate the oxidase, i.e. phosphorylation of p47^phox synergizes with AA in the oxidase activation. To test this possibility, we estimated the abilities of phosphorylated p47^phox to bind to p22^phox and to activate the oxidase in the presence of AA. As shown in Fig. 5, A and B, binding of phosphorylated p47^phox to p22^phox was enhanced by AA at 1–5 μM, where an unphosphorylated protein hardly or only slightly interacted with p22^phox. Furthermore, phosphorylated p47^phox was capable of supporting superoxide production more efficiently in the presence of 1–5 μM AA (Fig. 5C); AA shortened a lag time (Fig. 5D) and increased the maximal rate of the production (Fig. 5E). Thus PKC-mediated phosphorylation and AA synergistically induce a conformational change of p47^phox to a state capable of both binding to p22^phox and activating the oxidase.

As we have previously shown (22), p47-DC (amino acids 1–286), lacking the C-terminal region, activated the NADPH oxidase in the absence of AA, to an extent similar to the way phosphorylated p47^phox did (Fig. 5F). In p47-DC, the SH3 domains are partially unmasked because of the lack of interaction with the C-terminal target (amino acids 286–340) (Fig. 1E) as are the domains in phosphorylated p47^phox (Fig. 3A). The p47-DC-triggered activation of the oxidase was further activated by low concentrations (1–5 μM) of AA to an extent similar to the phosphorylated p47^phox-induced one (Fig. 5F). Thus p47-DC appears to functionally replace the phosphorylated p47^phox.

AA Cooperates with PKC to Induce Superoxide Production by Intact Human Neutrophils—To investigate whether AA and PKC-mediated phosphorylation of p47^phox also cooperate to activate the phagocyte NADPH oxidase in vivo, we stimulated human neutrophils with both AA and the PKC activator PMA in the presence of BPB, an inhibitor of PLA2. Although BPB appears to inhibit cellular events independent of PLA2 as well, it is well known that BPB blocks superoxide production as well as AA release in response to a variety of stimuli including PMA (14–17), whereas the inhibitor does not affect phosphorylation of p47^phox in PMA-stimulated neutrophils (16). Neutrophils produced only a trace amount of superoxide in response to PMA in the presence of BPB at 5 μM (Fig. 6A), where AA release is impaired, albeit phosphorylation of p47^phox is fully induced by PMA (16). As shown in Fig. 6B, the superoxide production was partially restored by the addition of AA. The restoration was observed at low concentrations of AA (1–5 μM), where AA alone failed to induce superoxide production (Fig. 6C). Thus AA and PMA synergistically activate the NADPH oxidase in neutrophils treated with the PLA2 inhibitor BPB.

**DISCUSSION**

Here we demonstrate that AA and phosphorylation synergistically induce a conformational change of p47^phox to a state capable of both interacting with p22^phox and activating the phagocyte NADPH oxidase in a cell-free system and propose a model for the mechanism underlying the synergism based on the present findings. In addition, we provide evidence that the synergism functions in the oxidase activation in whole cells.

**Conformational Change of p47^phox Induced by AA at High Concentrations**—In a resting conformation, p47^phox fails to bind to p22^phox, because the SH3 domains of p47^phox are masked mainly via an intramolecular interaction with the region C-terminal to the domains (amino acids 286–340). AA, at high concentrations (50–100 μM), induces direct interaction of p47^phox with p22^phox in parallel with activation of the oxidase (Fig. 1A). The induction likely involves, as a rate-limiting step, AA-evoked disruption of the intramolecular interaction in p47^phox, because AA disrupts the interaction between p47- (SH3)_2 and p47-(286–340) in a dose-dependent manner (Fig. 2,

FIG. 5. Synergism of AA and phosphorylation of p47^phox in interaction between p47^phox and p22^phox and activation of the NADPH oxidase in a cell-free system. A, phosphorylated or unphosphorylated GST-p47^phox (150 pmol) was incubated with MBP-p22-C (300 pmol) in the presence of the indicated concentration of AA and pulled down with glutathione-Sepharose-4B. Precipitated proteins were subjected to SDS-PAGE, followed by an immunoblot with anti-MBP antibodies. For details, see "Experimental Procedures." B, intensities of the immunoblot in A were quantified by an image analyzer, and the calculated ratio of bound MBP-p22-C to total MBP-p22-C was plotted. The values represent the mean ± S.E. of three independent experiments. C, the phagocyte NADPH oxidase was activated with phosphorylated or unphosphorylated GST-p47^phox (5 μg/ml), human neutrophil membranes (8.7 μg/ml), Rac2 (8.4 μg/ml) preloaded with GTP_S, and GST-p67-N (9 μg/ml) in the presence of 2.5 μM AA. Superoxide production was spectrophotometrically monitored by ferricytochrome c reduction. D, the phagocyte NADPH oxidase was activated with phosphorylated or unphosphorylated GST-p47^phox (5 μg/ml), human neutrophil membranes (8.7 μg/ml), Rac2 (8.4 μg/ml) preloaded with GTP_S, and GST-p67-N (9 μg/ml) in the presence of the indicated concentration of AA. Lag times were determined, as illustrated in C. The values represent the mean ± S.E. of three independent experiments. E, the phagocyte NADPH oxidase was activated under the conditions as in C, and superoxide production was determined as described under "Experimental Procedures." The values represent the mean ± S.E. of three independent experiments. F, the phagocyte NADPH oxidase was activated under the conditions as in C, except that GST-p47-F was used instead of GST-p47^phox. Superoxide production was determined as described under "Experimental Procedures." The values represent the mean ± S.E. of three independent experiments. SOD, superoxide dismutase.
AA and Phosphorylation Synergistically Activate p47phox

Synergism of AA and PMA in superoxide production by intact human neutrophils. A, human neutrophils (1 x 10^6 cells/ml) were preincubated for 5 min with BPB or Me_2SO and stimulated with 100 ng/ml PMA. Shown are spectrophotometric records of superoxide production monitored by ferricytochrome c reduction. B, neutrophils (1 x 10^6 cells/ml) were preincubated for 5 min with BPB and stimulated with 100 ng/ml PMA. After 1 min of PMA stimulation, AA (5 μM) was added to the suspension. A representative was shown from more than three independent experiments. C, neutrophils (1 x 10^6 cells/ml) were preincubated for 5 min with BPB and stimulated for 1 min with 100 ng/ml PMA followed by the addition of the indicated concentration of AA. The rate of superoxide production for the first 3 min was determined as described under "Experimental Procedures." The experiments have been repeated more than twice with similar results. SOD, superoxide dismutase.

A and C, which correlates with AA-induced binding of p47phox to p22^phox (Fig. 1A). On the other hand, the interaction between p47-(SH3)_2 and p22^phox is not affected by the addition of AA (Fig. 2B). These findings indicate that AA primarily disrupts the intramolecular interaction to render p47phox in a conformation capable of binding directly to p22phox and activating the oxidase.

Phosphorylation-induced Conformational Change of p47phox and Activation of the NADPH Oxidase—The present study shows that PKC-mediated phosphorylation of p47phox induces its interaction with p22phox, albeit to a lesser extent compared with the AA-induced one (Fig. 3E). Consistent with this, phosphorylated p47phox is capable of activating the NADPH oxidase under cell-free conditions (Fig. 4, A and B). Phosphorylation of serine residues in the intramolecular SH3 target region (amino acids 286–340) appears to play a crucial role in the oxidase activation, because substitution of the kinase-insensitive alanine for Ser-303/Ser-304 or Ser-328 results in a complete or partial loss of superoxide production, respectively, in our cell-free system (Fig. 4D). The results agree with the previous reports showing that the mutant p47phox with the S303A/S304A or S328A substitution is almost completely or partially inactive, respectively, when expressed in Epstein-Barr virus-transformed B cells (38, 39). Thus the activation mechanism of the oxidase in the present cell-free system appears to be at least analogous to that operating in whole cells.

The importance of phosphorylation of the SH3 target region in regulation of p47phox is also supported by a recent observation that p47phox carrying the replacement of these serines by aspartates, that mimic phosphorylated residues, activates the oxidase without AA in a cell-free system to a level similar to p47-DΔC (40). In addition, it has recently been reported that cell-free activation of the oxidase is inhibited by peptides derived from the SH3 target region of p47phox but not by phosphorylated ones (41).

Synergistic Activation of p47phox by Phosphorylation and Arachidonic Acid at Low Concentrations—The disruption of the SH3-mediated intramolecular interaction, by itself, is not sufficient for stable binding of p47phox to p22phox, PKC-mediated phosphorylation of the SH3 target region in p47phox results in a completely disrupted intramolecular interaction (Fig. 3A) but leads to a weak binding to p22phox (Fig. 3E), which is consistent with that activation by AA, and yields a greater production of superoxide than activation by phosphorylated p47phox (Fig. 4, A and B; and Refs. 32 and 33). Thus PKC-mediated phosphorylation of p47phox only partially replaces the effects of AA.

A C-terminally truncated form of p47phox, p47-DΔC (amino acids 1–286), can bind to p22phox via the SH3 domains that are partially unmasked because of a lack of their interaction with the C-terminal target (amino acids 286–340) as in phosphorylated p47phox. The binding of p47-DΔC to p22phox is enhanced by AA at lower concentrations than those required for the disruption of the intramolecular interaction with the C-terminal region (Fig. 1E). In addition, p47-DΔC appears to functionally replace phosphorylated p47phox, p47-DΔC activated the NADPH oxidase in the absence of AA, to an extent similar to what phosphorylated p47phox did, and the p47-DΔC-triggered activation is further activated by low concentrations (1–5 μM) of AA as well (Fig. 5F). Thus the AA-induced enhanced binding of p47-DΔC to p22phox appears to explain how AA at low concentrations increases both interaction of phosphorylated p47phox with p22phox (Fig. 5, A and B) and phosphorylated p47phox-evoked activation of the oxidase (Fig. 5E). The precise mechanism for the enhancement is presently unknown. It may be possible that there exists another intramolecular interaction of the SH3 domains with the N terminus of p47phox, containing a consensus SH3 target motif (6, 42) and that the second interaction is disrupted by a low concentration of AA.

A Proposed Model for Mechanism Whereby AA and Phosphorylation of p47phox Synergistically Activate the Phagocyte NADPH Oxidase—On the basis of the present findings described above, we propose a model for activation of the phagocyte NADPH oxidase in which AA synergizes with phosphorylation of p47phox (Fig. 7). Phosphorylation of p47phox leads to a disrupted intramolecular interaction between the SH3 domains and the C-terminal region, the latter of which contains phosphorylated serines. The phosphorylation-induced unmasking of the SH3 domains, however, is insufficient, probably because of another intramolecular interaction with the N-terminal region. Hence phosphorylation by itself acts only as a
weak activator of the NADPH oxidase. AA, at low concentrations, causes a further conformational change to completely expose the SH3 domains, which allows p47phox to fully interact with p22phox and activate the oxidase.

In Vivo Activation of the Phagocyte NADPH Oxidase—The synergistic mechanism appears to underlie in vivo activation of the phagocyte NADPH oxidase as well. The addition of AA induces superoxide production in whole cells as well as in a cell-free system (7–13). Because the relatively high concentration of AA is required in both cases, the physiological relevance of AA as a second messenger has been questioned. In addition, the amount of AA released under physiological conditions is likely insufficient to activate the oxidase by itself, because the calcium ionophore A23187 is known to be the strongest inducer of AA release in neutrophils but a poor agonist for superoxide production (43–45). Several lines of evidence, however, suggest involvement of PLA2, an enzyme that releases AA from membrane phospholipids upon cell stimulation. PLA2 inhibitors prevent superoxide production elicited by a variety of stimuli without affecting phosphorylation of p47phox (16). Furthermore, most importantly, in cytosolic PLA2-deficient phagocytic cells, PMA, a potent activator of the oxidase, fails to trigger superoxide production, although p47phox appears to be fully phosphorylated (17).

Phosphorylation of p47phox also seems required for the oxidase activation in vivo; the mutant p47phox carrying the double substitution S303A/S304A only marginally corrects the defect in superoxide production in Epstein-Barr virus-transformed p47phox−deficient B cells (38), whereas this mutant protein is capable of fully activating the oxidase in vitro in the presence of AA (38). On the other hand, agents such as GM-CSF and lipopolysaccharide, which are known to prime the oxidase activation, induce phosphorylation of p47phox without triggering superoxide production in human neutrophils (46–48).

These reported observations indicate that both AA released by cytosolic PLA2 and phosphorylation of p47phox are likely essential, but each of them by itself is insufficient for the oxidase activation in vivo. This is well explained by the present model in which both events synergistically activate p47phox, leading to superoxide production (Fig. 7). The present findings show that low concentrations of AA can activate the oxidase not only in vitro but also in vivo under the conditions where p47phox is phosphorylated (Figs. 5 and 6). It should be emphasized that the synergistic activation occurs at low, physiologically attainable (49), concentrations of AA (1–5 μM), which may argue against a conclusion that AA does not act as an in vivo activator of the oxidase, based on the requirement of high concentrations (50–100 μM) for activation by AA alone both in vivo and in vitro (18).

A recent study has shown that without AA phosphatidic acid fully activates the phagocyte NADPH oxidase in a cell-free system (50). This finding suggests that this phospholipid replaces AA to activate p47phox. It is presently unknown whether phosphatidic acid causes a conformational change of p47phox by the same mechanism as does AA and whether phosphatidic acid also synergizes with phosphorylation of p47phox in activation of the oxidase. These questions should be addressed in future studies.

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