Mechanism for Phosphorylation-induced Activation of the Phagocyte NADPH Oxidase Protein p47\textsuperscript{phox}

TRIPLE REPLACEMENT OF SERINES 303, 304, AND 328 WITH ASPARTATES DISRUPTS THE SH3 DOMAIN-MEDIATED INTRAMOLECULAR INTERACTION IN p47\textsuperscript{phox}, THEREBY ACTIVATING THE OXIDASE*

(Received for publication, July 8, 1999, and in revised form, August 26, 1999)

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Activation of the superoxide-producing phagocyte NADPH oxidase requires interaction between p47\textsuperscript{phox} and p22\textsuperscript{phox}, which is mediated via the SH3 domains of the former protein. This interaction is considered to be induced by exposure of the domains that are normally masked by an intramolecular interaction with the C-terminal region of p47\textsuperscript{phox}. Here we locate the intramolecular SH3-binding site at the region of amino acid residues 286–340, where Ser-303, Ser-304, and Ser-328 that are among several serines known to become phosphorylated on cell stimulation exist. Simultaneous replacement of the three serines in p47\textsuperscript{phox} with aspartates or glutamates, each mimicking phosphorylated residues, is sufficient for disruption of the intramolecular interaction and resultant access to p22\textsuperscript{phox}. The triply mutated proteins are also capable of activating the NADPH oxidase without in vitro activators such as arachidonate under cell-free conditions. In a whole-cell system where expression of the wild-type p47\textsuperscript{phox} reconstitutes the stimulus-dependent oxidase activity, substitution of the kinase-insensitive residue alanine for Ser-328 as well as for Ser-303/Ser-304 leads to a defective production of superoxide. These findings suggest that phosphorylation of the three serines in p47\textsuperscript{phox} induces a conformational change to a state accessible to p22\textsuperscript{phox}, thereby activating the NADPH oxidase.

Protein-protein interactions form the basis of a variety of cellular processes. The interactions often depend on modular domains that serve as specific protein-binding structures (reviewed in Refs. 1–4). Among them, SH3\textsuperscript{1} domains, found in a wide array of proteins involved in intracellular signal transduction and cytoskeletons, interact with proline-rich ligands via direct binding to the PX\textsuperscript{2}P motif (where P denotes proline residue; X denotes any amino acid residue; and Φ denotes a hydrophobic residue) (1–4). SH3-mediated interactions were initially considered to be constitutive, e.g. the adaptor protein Grb2 associates with the Ras activator Sos in a preformed heterodimeric complex, which is mediated via binding of the Grb2 SH3 domains to the C-terminal proline-rich tail of Sos. There exist, however, currently increasing examples in which SH3-mediated interactions are regulated (5–16), although molecular mechanisms underlying their regulation remain largely unknown.

The first example to be described as a regulatory SH3-mediated interaction is the one involved in the signaling system for activation of the superoxide-producing NADPH oxidase in phagocytes as well as B lymphocytes (5, 6). During phagocytosis or with appropriate stimuli, the phagocyte NADPH oxidase, dormant in resting cells, becomes activated to produce superoxide, a precursor of microbicidal oxidants (reviewed in Refs. 17–22). The significance of the enzyme in host defense is exemplified by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease, whose phagocytes are deficient in the superoxide-producing activity. The catalytic core of the oxidase is membrane-bound flavocytochrome b558 comprising the two subunits gp91\textsuperscript{phox} and p22\textsuperscript{phox}, that transfers electrons upon activation from NADPH to oxygen molecule. When cells are stimulated, the three cytosolic proteins p47\textsuperscript{phox}, p67\textsuperscript{phox}, and the small GTPase Rac, each indispensable for the oxidase activation, translocate to the membrane where they assemble with the cytochrome.

p47\textsuperscript{phox} harbors two SH3 domains, which specifically interact with the C-terminal cytoplasmic PRR of p22\textsuperscript{phox} upon activation (5, 6). This induced interaction plays a crucial role in activation of the NADPH oxidase; both the interaction and superoxide production are completely abrogated by replacement of the conserved Trp-193 in the N-terminal SH3 domain with Arg or by substitution of Gln for Pro-156 in the PRR of p22\textsuperscript{phox}, a mutation that occurs in a patient with chronic granulomatous disease (5, 6, 23–25). Since the full-length wild-type p47\textsuperscript{phox} in resting phagocytes or the one expressed in Escherichia coli or budding yeast is incapable of binding to p22\textsuperscript{phox}, a resting form of p47\textsuperscript{phox} is likely in a closed inactive conformation in which the SH3 domain is masked (5, 26). We and Leto et al. (5, 6) have previously proposed a model that the C-terminal region (residues 286–390) of p47\textsuperscript{phox} intramolecularly interacts with the SH3 domains to render this protein in the closed state, and, upon activation, the SH3 domains are unmasked to bind to the target p22\textsuperscript{phox}. Anionic amphiphiles such as arachidonate and SDS, activators of the oxidase in vitro (27), cause a conformational change of p47\textsuperscript{phox} to expose the SH3

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan, and grants from Kato Memorial Bioscience Foundation, Fukuoka Cancer Society, and Core Research for Evolutional Science and Technology of Japan Science and Technology Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: SH3, Src homology 3; PRR, proline-rich region; PMA, phorbol 12-myristate 13-acetate; fMLP, N-formyl-methionyl-leucyl-phenylalanine; EDT, Epstein-Barr virus; PCR, polymerase chain reaction; GST, glutathione S-transferase; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; GTP\textsubscript{7S}, guanosine 5′-3-O-(thio)-triphosphate.
domains, as suggested by analyses using an anti-SH3 monoclonal antibody (5) and tryptophan fluorescence spectroscopy (28). This “unmasking-masking” model for SH3-mediated regulatory interactions has been supported by a recent observation that a C-terminally truncated p47phox (p47ΔC; amino acid residues 1–286), in which the intramolecular interaction does not occur because of a lack of the SH3 target, is capable of both binding to p22phox and activating the oxidase in the absence of the amphiphile activators (26).

It is well established that, upon cell stimulation, p47phox becomes extensively phosphorylated (29–31). An intensive study by Babior’s group (32) has revealed that 9 to 10 serine residues within the C-terminal region of p47phox (Ser-303, Ser-304, and Ser-328) are phosphorylated when human neutrophils become extensively phosphorylated because the mutant p47phox carrying the double substitution S303A/S304A only marginally corrects the defect in superoxide production in EBV-transformed p47phox-deficient B cells (33). However, the molecular link between the phosphorylation event and the oxidase activation remains to be elucidated.

To address this question, we focused on relationship between phosphorylation of p47phox and a conformational change that leads to the oxidase activation. As an initial step of the analyses, we replaced the serines of p47phox with aspartates or glutamates, each mimicking phosphorylated residues in various proteins (14, 34–36), and we tested the effects of replacements on the SH3-mediated intramolecular and intermolecular interactions. The experiments reveal that simultaneous replacement of Ser-303, Ser-304, and Ser-328 is sufficient for disruption of the intramolecular interaction and resultant access of the SH3 domains to p22phox. Furthermore, the triply mutated p47phox can activate the oxidase in vitro without the amphiphiles. Substitution of alanine for Ser-328 as well as for Ser-303/Ser-304 results in a defective production of superoxide in vivo. Thus phosphorylation of the three serines of p47phox induces a conformational change to a state accessible to p22phox, thereby activating the NADPH oxidase.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The DNA fragments encoding the full-length of p47phox (p47-F; amino acid residues 1–390), p47-(SH3)2-(154–286), p47-(SH3)(N 154–219), p47-(SH3)(C 223–286), p47-(1–286), p47-(1–314), p47-(1–327), and p47-(1–340) were amplified from a cloned cDNA encoding human p47phox by PCR using specific primers and ligated to pACT2 (CLONTECH) and pGEX-2T (Amersham Pharmacia Biotech). Similarly, the DNA fragment encoding p47-(286–302), p47-(286–314), p47-(286–327), p47-(286–340), and p47-C-(286–390) were generated by PCR and cloned into pMALc2 (New England Biolabs). Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis, and the mutated fragments were cloned into the indicated vectors. The DNA fragments encoding the C-terminal cytoplasmic region of p22phox, p22C-(132–195) and its mutant p22C-(152K6) were prepared as previously described (25, 24) and ligated to pMALc2 and pGBT9 (CLONTECH). All the constructs were sequenced to confirm their identities.

Two-hybrid Experiments—Various combinations between pGBT9 and pACT2 plasmids were co-transformed into yeast Y190 cells containing HIS3 and lacZ reporter genes using a lithium-acetate method (37). Following the selection for Trp+ and Leu+ phenotype, the transformants were tested for their ability to grow on plates lacking histidine supplemented with 25 mM 3-aminotriazole to suppress the background growth of activation of lacZ reporter was examined by the β-galactosidase filter assay according to the manufacturer’s recommendation (CLONTECH).

An in Vitro Binding Assay Using Purified Proteins—Proteins fused to GST or to MBP were expressed in E. coli strain BL21 and purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) or amylose resin (New England Biolabs), respectively, according to the manufacturer’s protocols. For in vitro pull-down binding assays, a pair of a GST fusion and an MBP-tagged protein were mixed in 500 μl of phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4, and 1.47 mM KH2PO4) containing 1% Triton X-100 and incubated for 30 min at 4 °C. A slurry of glutathione-Sepharose 4B or amylose resin was subsequently added, followed by further incubation for 30 min at 4 °C. After washing three times with phosphate-buffered saline, proteins were eluted from glutathione-Sepharose 4B or amylose resin, with 5 mM glutathione in 50 mM Tris-HCl (pH 8.0) or with 10 mM amylase in 50 mM Tris-HCl (pH 8.0), respectively. The eluates were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

Phosphorylation as a Switch for SH3-mediated Interactions—The membrane fraction of human neutrophils was prepared as described previously (5, 24, 26). The membranes (10 μg of protein/ml) were mixed with the indicated concentrations of the wild-type or mutant p47phox fused to GST, an N-terminal fragment of p67phox (p67-N; amino acids 1–242) as a GST fusion protein (10 μg/ml), and His-tagged Rac2 (10 μg/ml) preloaded with 100 μM GTPγS, followed by incubation with or without SDS (100 μM) for 2.5 min at room temperature in 100 mM potassium phosphate, pH 7.6, containing 75 μM cytochrome c, 10 μM FAD, 1.0 mM EGTA, 1.0 mM MgCl2, and 1.0 mM Na3VO4. The reaction was initiated by addition of NADPH (250 μM) 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 to 540 nm with a dual-wavelength spectrophotometer (Hitachi 557) (5, 24, 26).

Activation of the NADPH Oxidase in the Whole-cell System—We used a retroviral vector system, pSXLClpA, that utilizes an internal ribosome entry site fragment of encephalomyocarditis virus (38) to transduce the gp91phox gene into the leukemia cell line K562 that expresses p22phox but not gp91phox (39). Cells highly expressing gp91phox were selected using FACS scan with the monoclonal antibody 7D5 to detect functional cytochrome b558 comprising the two subunits gp91phox and p22phox (40). A bicistronic retrovirus vector encoding a human multi-drug resistance gene (MDR1) and the p67phox gene (pHa-MDR-IRESP67) (41) was further transduced to the stably transduced gp91phox-expressing K562 cells. The doubly transduced cells were selected with 4 mg/ml G418, expanded in a drug-free medium, and used for the following experiments.

Complementary DNAs encoding the full-length of the wild-type and mutant p47phox carrying the S328A or S303A/S304A substitution were subcloned into pREP4 (Invitrogen), which were transfected by electroporation and the K562 cells that stably express both gp91phox and p67phox. The K562 cells (2 × 106 cells/ml) were electroporated in the presence of 100 μg/ml of wild-type or mutant form of p47phox (100 μg/ml), 960 microfarads using Gene Pulser (Bio-Rad). To obtain stable transformants, cells were selected for over 30 days with 100 mg/ml hygromycin B. For detection of p47phox and p67phox, K562 cells (1 × 106 cells) were lysed by sonication, and the sonicates were applied to 10% SDS-PAGE. Proteins were transferred to a polyvinyliden difluoride membrane (Millipore) and probed with anti-p47phox and anti-p67phox monoclonal antibodies (both from Transduction Laboratories). The blots were developed using ECL-plus (Amersham Pharmacia Biotech) to visualize the antibodies.

Superoxide production by K562 cells (1 × 106 cells) expressing the wild-type or mutant p47phox was determined as superoxide dismutase-inhibitable chemiluminescence detected with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) as described by de Mendez et al. (39). After the addition of the enhanced luminol-based substrate, the cells were stimulated with 200 ng/ml PMA. The chemiluminescence was assayed using luminometer (Auto Lumat LB953; EG & G Berthold).

RESULTS

C-terminally Truncated p47phox Is Capable of Binding to p22phox—The two SH3 domains of p47phox are tandemly arranged in the central portion in the primary sequence: The N-terminal residues amino acid residues 154–219 (SH3(N)) and the C-terminal one of residues 223–286 (SH3(C)) (Fig. 1). We have previously shown that the C-terminal region of gp91phox (amino acids 286–390) interacts with the SH3 domains in an intramolecular fashion, which likely prevents the domains from binding to p22phox (5, 26). To map precisely the region responsible for the prevention, we expressed and purified the full-length p47phox (p47-F; amino acids 1–390) and a series of C-terminally truncated p47phox (p47-(1–340), p47-(1–327), p47-
Phosphorylation as a Switch for SH3-mediated Interactions

The Region of Amino Acids 286–340 in p47phox Is Responsible for the Intramolecular Interaction with the SH3 Domains—The findings described above suggest that the fragment of amino acids 286–314 plays an important role in blockade of the SH3-mediated interaction between p47phox and p22phox, probably by intramolecular binding to the SH3 domains. To test this possibility, we expressed a variety of C-terminally truncated p47phox proteins (Fig. 2A), and we tested their ability to bind to the C-terminal cytoplasmic tail of p22phox (amino acids 132–195), termed p22-C. As shown in Fig. 2B, GST-p47-ΔC-(1–286) was fully precipitated with amylase resin coupled to MBP-p22-C, whereas GST alone was hardly recovered. The interaction seems to be mediated via the SH3 domains of p47phox, since p47-ΔC was not recovered when resins were coupled to MBP alone or a mutant p22-C carrying the P156Q substitution (data not shown). p47-(1–302) also bound to MBP-p22-C but to a lesser extent (Fig. 2B). On the other hand, neither p47-(1–314) nor p47phox proteins with a shorter deletion could interact with p22-C. Although there were substantial small protein fragments in the sample of p47-(1–314) (Fig. 2A), the possibility that they act as an inhibitor in binding can be excluded, since the sample of p47-(1–314) did not affect the interaction of p22-C with p47-ΔC or with p47-(1–302) (data not shown). These results were confirmed by an in vitro binding assay using the yeast two-hybrid system; both histidine-independent growth and β-galactosidase activity were observed, only when the yeast Y190 cells were co-transformed with both the DNA-binding domain fusion vector pGBT9 encoding p22-C (pGBT9-p22-C) and the transactivation domain fusion vector pACT2 encoding p47-ΔC or p47-(1–302) (data not shown).

The Tandem SH3 Domains Synergistically Interact with the Region of Amino Acids 286–340 in p47phox—To clarify the role for each of the two SH3 domains of p47phox in the intramolecular interaction, we compared the ability to bind to the target region of amino acids 286–340 among p47-SH3(N), p47-SH3(C), and p47-SH3(NC). As shown in Fig. 4A, a negligible binding activity was observed when p47-SH3(N) or p47-SH3(C) was used instead of p47-SH3(NC). Thus the tandem SH3 domains appear to interact synergistically with the intramolecular target site to keep p47phox in a closed inactive conformation.

The Stretch PPRR of Amino Acids 299–302 Plays an Essential Role in the Intramolecular Interaction of p47phox—Structural analyses of several SH3-ligand complexes have revealed that SH3 domains bind to a proline-rich region in the polyproline II (PPII) helix conformation with extremely high preference to the sequence RX(Phi)XX(P type I ligand) and/or ΦXΦXXR (type II ligand), and thus ΦXXΦ is generally accepted as the target motif of SH3 domains accordingly (43–46). Such a ΦXXΦ motif, however, is absent in the intramolecular SH3 target region of p47phox (amino acids 286–340); there exist only two proline residues at positions 299 and 300 (Fig. 1). Instead, the sequence of 299–302, PPRR, that is present in the region minimally required for the intramolecular interaction (amino acids 286–314), turns out to be a remnant of the type II
SH3 ligand and is thereby expected to play a role in the interaction with the SH3 domains (Fig. 1). To test this possibility, we substituted glutamines for Pro-299 and Pro-300 (P299Q/P300Q) or glutamates for Arg-301 and Arg-302 (R301E/R302E). A weak binding of p47(286–314) to p47-(SH3)2 was abolished by these substitutions (Fig. 3). The dual role for the SH3 domains (Fig. 3). The dual role for the SH3 domains (residues 43–46) in the interaction with the SH3 domains is expected to be disrupted. As shown in Fig. 5, A and B, proteins with these substitutions were capable of interacting with p22(phox). The results were consistent with those by the pull-down binding assay using purified proteins (Fig. 5B). MBP-p22-C directly interacted with the mutated proteins but not with the wild-type one (Fig. 5C). Taken together, we concluded that p47(phox) becomes inaccessible to p22(phox) solely by disrupting the intramolecular interaction.

**Triple Replacement of Ser-303, Ser-304, and Ser-328 with Aspartates or Glutamates Is Sufficient for p47(phox) to Interact with p22(phox)**—It is known that, upon cell stimulation, p47(phox) becomes extensively phosphorylated at more than nine serine residues of the C-terminal quarter (32). They include five serines present in the region required for the stable intramolecular interaction with the p47(phox) SH3 domains (residues 286–340) as follows: Ser-303, Ser-304, and Ser-328. Among these serine residues, we initially focused on Ser-303 and Ser-304. Since these residues are intensively phosphorylated in stimulated cells (32) and exist just adjacent to the SH3-binding PPRR sequence of 299–302, phosphorylation of these serines may disrupt the SH3-mediated intramolecular interaction, thereby activating p47(phox). To test this possibility,
we replaced the serines by aspartates or glutamates, which are known to mimic phosphorylated residues in various proteins (14, 34–36). Unexpectedly, a full-length p47phox carrying the double substitution of aspartates for Ser-303 and Ser-304, designated p47-F (S303D/S304D), could not interact with p22-C in the two-hybrid experiment (Fig. 6A), raising the possibility that additional modification may be required for conversion of p47phox to a state accessible to p22phox.

To identify other serine residues to be modified, we truncated p47phox (S303D/S304D) from the C terminus. The two-hybrid experiment revealed that p47phox carrying the triple substitution did interact with p22-C, whereas those carrying
TABLE I

| Interaction between full-length p47<sup>phox</sup> carrying replacement of serines and p22<sup>phox</sup> in the yeast two-hybrid system |
|---------------------------------------------------------------|
| p47<sup>phox</sup> | Histidine-independent growth |
|------------------|-----------------------------|
| Wild-type | − |
| S303D/S304D | − |
| S303D/S328D | − |
| S304D/S328D | − |
| S303D/S304D/S328D | + |
| S303D/S304D/S315D | − |
| S303D/S304D/S320D | − |
| S315D/S328D | − |
| S320D/S328D | − |
| S303D/S304D/S315D/S320D | − |
| S315D/S320D/S328D | − |
| S303D/S304D/S315D/S326D | + |
| S303D/S304D/S320D/S326D | + |
| S303D/S304D/S315D/S320D/S328D | + |
| S345D/S348D | − |
| S303D/S304D/S345D/S348D | − |
| S320D/S345D/S348D | − |
| S303D/S304D/S328D/S345D/S348D | + |
| S359D/S370D/S379D | − |
| S303D/S304D/S328D/S359D/S370D/S379D | + |

The single substitution of S328D failed to bind to p22-C. The interaction seems to be specific, since the triply mutated protein was incapable of binding to p22-C (P156Q) (data not shown). The same results were obtained when serines were replaced with glutamates, also mimicking phosphorylated residues, instead of aspartates (Fig. 6B). On the other hand, alanines could not replace aspartates or glutamates (Fig. 6B).

All the three serines (Ser-303, Ser-304, and Ser-328) to be replaced are likely required for p47<sup>phox</sup> to interact with p22<sup>phox</sup>, since neither p47-F (S303D/S328D) nor p47-F (S304D/S328D) was capable of interacting with p22-C (Table I). In addition, replacement of Ser-315, Ser-320, or both, instead of Ser-328, in p47-F (S303D/S304D/S326D) did not lead to interaction with p22-C (Table I). Furthermore, any interactions could not be promoted by substitutions of aspartates for serines that lie outside of the SH3-targeted region: double substitution for Ser-345 and Ser-348 (Table I), the two serines that can be phosphorylated by the MAP kinases ERK and p38 (47, 48), or triple substitution for the three C-terminal serines at positions 359, 370, and 379 (Table I). Thus the simultaneous substitution for Ser-303, Ser-304, and Ser-328 appears to be sufficient for promoting the interaction with p22<sup>phox</sup>.

**Tripal Rephcement of Ser-303, Ser-304, and Ser-328**

Is Required for p47<sup>phox</sup> to Interact with p22<sup>phox</sup>—To clarify the effect of each substitution of aspartate for Ser-303, Ser-304, or Ser-328, we constructed a mutant p47<sup>phox</sup>, designated p47-F (S303–379D), in which aspartate replaces all 10 serines to be phosphorylated in stimulated cells (Ser-303, Ser-304, Ser-315, Ser-320, Ser-328, Ser-345, Ser-348, Ser-359, Ser-370, and Ser-379) (32), and those carrying substitutions for all but one serine among Ser-303, Ser-304, and Ser-328. As shown in Fig. 6C, the protein p47-F (S303–379D) binds to p22-C, whereas mutant proteins containing unreplaced Ser-303, Ser-304, or Ser-328 all failed to interact with the intermolecular target. Thus the three serine residues must be simultaneously replaced for p47<sup>phox</sup> to interact with p22<sup>phox</sup>.

**Triple Replacement of Ser-303, Ser-304, and Ser-328 Results**

in Disruption of the SH3-mediated Intramolecular Interaction of p47<sup>phox</sup>—It seems likely that the binding of the triply mutated p47<sup>phox</sup> (S303D/S304D/S328D) to p22<sup>phox</sup> resulted from a defect of the SH3-mediated intramolecular interaction. To confirm this, we prepared the SH3-targeted fragment (amino acids 286–340) with the substitution S303D/S304D and/or S328D, and we tested their ability to interact with p47<sup>SH3/2</sup>. The fragment carrying the S303D/S304D or S328D substitution bound to p47<sup>SH3/2</sup> more weakly than the wild-type one did (Fig. 7). The triple substitution for Ser-303, Ser-304, and Ser-328 resulted in a completely defective interaction (Fig. 7), which is consistent with the finding that the only triply mutated p47<sup>phox</sup> gains access to p22<sup>phox</sup> (Figs. 5C and 6A). Taken together, simultaneous phosphorylation of Ser-303, Ser-304, and Ser-328 of p47<sup>phox</sup> appears to primarily disrupt the SH3-mediated intramolecular interaction, thereby leading to the interaction of the unmasked SH3 domain with p22<sup>phox</sup>. (Fig. 7).

**Mutant p47<sup>phox</sup> Proteins That Are Accessible to p22<sup>phox</sup> Can Support Superoxide Production in an Anionic Amphiphile-independent Manner under Cell-free Activation Conditions of the NADPH Oxidase**—As described above, a mutant p47<sup>phox</sup> carrying the triple replacement of Ser-303, Ser-304, and Ser-328 with aspartates, mimicking a phosphorylated form, is in a conformation capable of binding to p22<sup>phox</sup>. We next tested how this mutant protein serves in activation of the phagocyte NADPH oxidase.

The NADPH oxidase can be activated by anionic amphiphiles such as arachidonate and SDS in a cell-free system reconstituted with human neutrophil membranes that contain a high amount of the catalytic core cytochrome <i>b</i><sub>558</sub> and three cytosolic proteins: p47<sup>phox</sup>, p67<sup>phox</sup>, and the small GTPase Rac1/2 in the GTP-bound state (24, 26). We have recently shown that, even without the amphiphiles, the oxidase can be activated in <i>vitro</i> by p47<sup>phox</sup> and p67<sup>phox</sup>, both in C-terminally truncated forms, in the presence of the GTP-bound Rac (26). When the full-length p47<sup>phox</sup> (p47-F) is used instead of the truncated p47<sup>phox</sup>, p47<sup>ΔC</sup> (residues 1–286), the activation absolutely requires the amphiphiles (Ref. 26 and Fig. 8). The finding implies that p47<sup>phox</sup> is a target of the amphiphiles and that p47<sup>ΔC</sup> serves as an active form of p47<sup>phox</sup>.

By using this system, we tested whether mutant p47-F proteins mimicking a phosphorylated form can replace p47<sup>ΔC</sup> to activate the oxidase in <i>vitro</i> without the amphiphiles. As shown in Fig. 8A, p47-F (S303D/S304D/S328D) was capable of supporting superoxide production in the anionic amphiphile-independent system for the oxidase activation, although higher concentrations were required for fully activating the oxidase.
Phosphorylation as a Switch for SH3-mediated Interactions

The SH3-mediated Intramolecular Interaction as the Major Determinant for Active and Inactive Conformations of \( p47^{phox} \)—We have previously shown that activation of the phagocyte NADPH oxidase absolutely requires interaction between \( p47^{phox} \) and \( p22^{phox} \) which is mediated via the SH3 domains of the former protein (5, 24). This interaction is considered to be induced by exposure of the domains that are normally masked by an intramolecular interaction with the C-terminal region of \( p47^{phox} \) (5, 24, 26). However, properties of the intramolecular interaction have remained largely unknown as follows: which region acts as the SH3 target; which SH3

The order of the potency to activate the oxidase (Fig. 8A) is identical with that to bind to \( p22^{phox} \) (Fig. 5C: \( p47^{p299Q/P300Q} \) > \( p47^{F} \) (R301E/R302E) > \( p47^{F} \) (S303D/S304D/S328D) > \( p47^{F} \) (S328D) = \( p47^{F} \) (S303D/S304D)). Thus the binding of \( p47^{p299Q} \) to \( p22^{phox} \) is a rate-limiting step in the oxidase activation. Taken together with the other results obtained here, we conclude that “activation of the molecule \( p47^{phox} \)” is achieved by gaining the accessibility to \( p22^{phox} \) via disrupting the SH3-mediated intramolecular interaction.

The S328A or S303A/S304A Substitution in \( p47^{phox} \) Results in Defective Activation of the NADPH Oxidase in a Whole-cell System—The present observations suggest that phosphorylation of Ser-328 as well as Ser-303 and Ser-304 of \( p47^{phox} \) causes a conformational change to a state accessible to \( p22^{phox} \), thereby activating the phagocyte NADPH oxidase. To investigate the role of the phosphorylation at a cell level, we have developed a whole-cell system of the K562 leukemic cell line. The cells are known to express Rac1/2 and a low level of endogenous \( p22^{phox} \) and to require expression of the other three oxidase factors (\( gp91^{phox} \), \( p47^{phox} \), and \( p67^{phox} \)) to exhibit superoxide production in response to PMA (39). To explore the function of \( p47^{phox} \), we transduced K562 cells for stable expression of \( gp91^{phox} \) and \( p67^{phox} \) using retroviral vectors encoding the proteins. The transduced cells expressed functional cytochrome \( b_{558} \) comprising the two subunits \( gp91^{phox} \) and \( p22^{phox} \) (data not shown; see “Experimental Procedures”) and \( p67^{phox} \) (Fig. 9A).

The K562 cells expressing both cytochrome \( b_{558} \) and \( p67^{phox} \) were subsequently transfected with the episomal vector \( pREP4 \) that contained cDNA encoding the full-length wild-type \( p47^{phox} \) (\( p47^{F} \) or full-length mutant proteins carrying replacement of serines by alanine, a residue that does not become phosphorylated, namely \( p47^{F} \) (S328A) and \( p47^{F} \) (S303A/S304A). The wild-type \( p47^{phox} \)-expressing cells fully produced superoxide when stimulated with PMA (Fig. 9, B and C). On the other hand, in the cells transfected with the \( p47^{F} \) (S328A) cDNA, the stimulant induced superoxide production but to a much lesser extent (Fig. 9, B and C), although the \( p47^{phox} \) protein was expressed at a similar level to the wild-type one in the control cells (Fig. 9A). Only a marginal production of superoxide was also detected upon stimulation in the cells expressing \( p47^{F} \) (S303A/S304A), consistent with the previous report showing that this mutant protein is essentially inactive when expressed in EBV-transformed \( p47^{phox} \)-deficient B cells (33). These substitutions unlikely lead to a loss of the ability to activate the oxidase, since bacterially expressed \( p47^{phox} \) with the S303A/S304A and S328A substitutions were both capable of supporting superoxide production in the amphiphile-dependent cell-free activation system in the same dose-dependent manner as the wild-type \( p47^{phox} \) (data not shown). Thus the S328A as well as S303A/S304A substitution of \( p47^{phox} \) resulted in a drastically decreased activation of the NADPH oxidase under the whole-cell conditions, indicating that phosphorylation of the three serines plays a crucial role.

DISCUSSION

The SH3-mediated Intramolecular Interaction as the Major Determinant for Active and Inactive Conformations of \( p47^{phox} \)—We have previously shown that activation of the phagocyte NADPH oxidase absolutely requires interaction between \( p47^{phox} \) and \( p22^{phox} \) which is mediated via the SH3 domains of the former protein (5, 24). This interaction is considered to be induced by exposure of the domains that are normally masked by an intramolecular interaction with the C-terminal region of \( p47^{phox} \) (5, 24, 26). However, properties of the intramolecular interaction have remained largely unknown as follows: which region acts as the SH3 target; which SH3

compared with \( p47^{F} \) (S303E/S304E/S328E) and \( p47^{F} \) (S303–379) were both active as \( p47^{F} \) (S303D/S304D/S328D) in the oxidase activation (data not shown). On the other hand, \( p47^{F} \) (S303D/S304D) or \( p47^{F} \) (S328D), each lacking the \( p22^{phox} \) binding activity (Fig. 5C), was incapable of activating the oxidase without amphiphiles (Fig. 8A), although these mutant proteins are as active as the wild-type \( p47^{phox} \) in the presence of the amphiphile activator SDS (Fig. 8B). Thus triple replacement of Ser-303, Ser-304, and Ser-328 renders \( p47^{phox} \) in a conformation capable of not only binding to \( p22^{phox} \) but also activating the NADPH oxidase.

These experiments also show that the ability of \( p47^{phox} \) to activate the NADPH oxidase in the amphiphile-independent system appears to be parallel with that to bind to \( p22^{phox} \). We next tested the ability of full-length mutant proteins carrying substitutions in the core intramolecular binding site for the SH3 domains of \( p47^{phox} \), \( p47^{F} \) (P299Q/P300Q) and \( p47^{F} \) (R301E/R302E), both of which were capable of interacting with \( p22^{phox} \) (Fig. 5). As shown in Fig. 8A, these proteins could activate the oxidase in an amphiphile-independent manner.
domain is involved; and, most importantly, how the interaction functions, and how it is regulated.

In this study, we locate the intramolecular SH3-binding site at the region of amino acids 286–340. The fragment of amino acids 286–314 is essential for a minimal interaction, whereas its C-terminal one (amino acids 315–340) is further required for a stable association (Fig. 3B). The SH3-binding site, somewhat to our surprise, lacks the canonical SH3 target motif PXFΦP; there exist only two proline residues at positions 299 and 300 in the region of amino acids 286–340 (Fig. 1). The present results show that the PPRR stretch (amino acids 299–302), a remnant of the type II SH3 ligand ΦPXΦPXR, likely conforms the binding core, since the interaction is completely abolished by the P299Q/P300Q substitution in both the minimal and full-length SH3 target (amino acids 286–314 and 286–340, respectively) (Fig. 3C). In addition, full-length mutant proteins of p47phox, P299Q/P300Q and R301E/R302E, are capable of not only binding to the intermolecular SH3 target p22phox (Fig. 5, A and C) but also activating the NADPH oxidase under cell-free conditions without the amphiphile activators (Fig. 8A). These observations establish that the SH3-mediated intramolecular interaction is the major determinant for keeping p47phox in a closed inactive conformation.

The interaction of p47phox with p22phox appears to be a rate-limiting step in the oxidase activation, as indicated by the observation that the p22phox binding activity of various mutant p47phox is completely parallel with their ability to activate the oxidase (Fig. 8A). Thus a conformation capable of engaging p22phox, resulting from disruption of the SH3-mediated intramolecular interaction, represents an active state of p47phox.

**Reason for the Two SH3 Domains to Be Tandemly Arrayed in p47phox:** For the Refined Regulation of the Conformational Change?—The intramolecular interaction that determines active and inactive conformation of p47phox requires both SH3 domains. The N-terminal SH3 domain of p47phox (amino acids 154–219) appears to contact directly with the PP RR stretch (amino acids 299–302), because the intramolecular interaction is abrogated by the substitution of Arg for Trp-193, the conserved residue among all SH3 domains that is expected to interact directly with a proline of the target (43–46), not by the corresponding mutation in the C-terminal SH3 domain (SH3(C)) (Fig. 4B). In addition to the core stretch, its C-terminal flanking region of about 40 residues is required for a stable SH3-mediated intramolecular interaction in p47phox. The allosteric effect of the region outside the binding core may suggest a role for SH3(C) as a binding partner for the extra core region, since SH3(C) also participates in the interaction, i.e. the two SH3 domains synergistically bind to the fragment of amino acids 286–340 (Fig. 4A). A similar mechanism underlies regulation of the Src family of protein kinases, in which the tandem SH3 and SH2 domains synergistically keep the enzyme in a closed inactive conformation via intramolecular interactions; the SH3 domain interacts with the linker between the SH2 and catalytic domains, whereas the SH2 domain binds to the phosphotyrosine-containing C-terminal tail of the kinase in a resting state (49, 50). Interestingly, the intramolecular SH3 target in Src lacks the PXΦP motif as well (49). Such a synergism may occur in p47phox, where the tandemly arrayed SH3 domains likely contribute to a refined regulation of the intramolecular interaction-dependent conformational change. This may explain why the tandem SH3 domains are present in p47phox.

**Triple Replacement of Ser-303, Ser-304, and Ser-328 in p47phox with Aspartates, a Mutation That Is Sufficient for Disruption of the SH3-mediated Intramolecular Interaction, Induction of Binding to p22phox and Activation of the NADPH Oxidase**—It is well established that stimulation of human neutrophils leads to extensive phosphorylation of p47phox in parallel with superoxide production (29–31). However, it has remained unknown about a molecular link between the phosphorylation event and activation of the phagocyte NADPH oxidase.

Here we demonstrate that simultaneous replacement of Ser-303, Ser-304, and Ser-328 in p47phox with aspartates or glutamates, each mimicking phosphorylated residues (14, 44–46), is sufficient for disruption of the SH3-mediated intramolecular interaction and resultant access of the unmasked SH3 domains to p22phox. The three serine residues, all being present in the intramolecular SH3 target site (amino acids 286–340), are known to become intensively phosphorylated when human
neutrophils are stimulated with PMA or fMLP (32). The triply mutated p47\textsubscript{phox} is considered to be in an active conformation, since it activates the phagocyte NADPH oxidase under cell-free conditions in a manner independent of the anionic amphiphilic activators (Fig. 8A). On the other hand, mutant p47\textsubscript{phox} with either S328D or S303D/S304D substitution is inactive in the cell-free system. Thus simultaneous phosphorylation of Ser-303, Ser-304, and Ser-328 in p47\textsubscript{phox} likely functions as a switch from a closed inactive conformation to a state capable of both binding to p22\textsubscript{phox} and activating the oxidase. The requirement for phosphorylation of the three serines is supported by the observation that substitution of the kinase-insensitive residue alanine for Ser-328 as well as for both Ser-303 and Ser-304 results in defective production of superoxide in PMA-stimulated cells (Fig. 9). Thus phosphorylation of Ser-303, Ser-304, and Ser-328 appears to primarily disrupt the intermolecular interaction to activate p47\textsubscript{phox}.

Role for Phosphorylation of Ser-328 as Well as Ser-303 and Ser-304 in p47\textsubscript{phox}—A recent study has suggested the importance of phosphorylation at serines 303 and 304 in the oxidase activation; the double mutant p47\textsubscript{phox} S303A/S304A is much less active than the wild-type one when expressed in EBV-transformed p47\textsubscript{phox}-deficient B cells (33). It has remained, however, unknown what is induced in p47\textsubscript{phox} carrying these phosphorylated residues. The present results show that phosphorylation of Ser-303 and Ser-304 is likely required for disruption of the SH3-mediated intramolecular interaction. In addition to both serines, Ser-328 also appears to be necessarily phosphorylated for activation of p47\textsubscript{phox}, since the S282A substitution results in defective activation of the oxidase in \textit{vivo} (Fig. 9). The strict requirement for the three serines to be phosphorylated is also supported by the finding that a protein carrying these residues to be replaced with aspartates (Fig. 6C).

Role for Phosphorylation of Other Serines in p47\textsubscript{phox}—It has been also suggested that a phosphorylated serine at position 359 or 370 participates in the oxidase activation by facilitating phosphorylation of the remaining serines (51). The two serine residues lie outside the intramolecular SH3 binding region of amino acids 286–340. In addition, a full-length p47\textsubscript{phox} carrying the S359D/S370D/S379D substitution fails to interact with p22\textsubscript{phox} (Table I), whereas the triple replacement of Ser-303, Ser-304, and Ser-328, all being present in the SH3 target site, with aspartates or glutamates, is sufficient for both disrupting the interaction and binding to p22\textsubscript{phox} (Figs. 5 and 6 and Table I). Serines 359 and 370 are located just N- and C-terminally to the PRR of p47\textsubscript{phox} (amino acids 360–369; KPQPAVPPRP), respectively. The PRR is constitutively occupied by the C-terminal SH3 domain of p67\textsubscript{phox} in a binding that occurs in a manner independent of the SH3-mediated intramolecular interaction in p47\textsubscript{phox} (26). Thus both Ser-359 and Ser-370 are likely sequestered from the intramolecularly interacting moiety of p47\textsubscript{phox}, and phosphorylation of these residues does not appear to be directly involved in disruption of the intramolecular interaction.

A Model for Phosphorylation-dependent Activation of p47\textsubscript{phox}—Based on the present findings, here we propose a model that phosphorylation of p47\textsubscript{phox} induces a conformational change to a state accessible to p22\textsubscript{phox}, thereby activating the NADPH oxidase (Fig. 10). In a resting state, p47\textsubscript{phox} is folded in a closed inactive conformation by an intramolecular interaction that is synergistically mediated via the tandem SH3 domains. Phosphorylation of Ser-303, Ser-304, and Ser-328 primarily disrupts the SH3-mediated intramolecular interaction and binding to p22\textsubscript{phox}, secondary to a state capable of activating the oxidase. The requirement for phosphorylation of Ser-303, Ser-304, and Ser-328 results in defective production of superoxide in PMA-stimulated cells (Fig. 9). Thus phosphorylation of Ser-303, Ser-304, and Ser-328 appears to primarily disrupt the intermolecular interaction to activate p47\textsubscript{phox}.

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FIG. 10. A model for phosphorylation-dependent activation of p47\textsubscript{phox}. In a resting state, p47\textsubscript{phox} is folded in a closed inactive conformation by the intramolecular interactions via the two SH3 domains. Simultaneous phosphorylation of Ser-303, Ser-304, and Ser-328 disrupts the intramolecular interaction. The disruption renders p47\textsubscript{phox} in an open conformation capable of interacting with p22\textsubscript{phox} and thereby activating the NADPH oxidase. For details, see text.
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