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吾郷, 哲朗
九州大学医学系研究科内科系専攻

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

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Tetsuro Ago‡, Hiroyuki Nunoi§, Takashi Ito¶, and Hideki Sumimoto‡‖

From the ‡Department of Molecular and Structural Biology, Kyushu University Graduate School of Medical Science, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, the §Department of Pediatrics, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-8556, and the ¶Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan

Activation of the superoxide-producing phagocyte NADPH oxidase requires interaction between p47^{phox} and p22^{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^{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 upon cell stimulation exist. Simultaneous replacement of the three serines in p47^{phox} with aspartates or glutamates, each mimicking phosphorylated residues, is sufficient for disruption of the intramolecular interaction and resultant access to p22^{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^{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^{phox} induces a conformational change to a state accessible to p22^{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[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 P$X\Phi$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 b_{558}, comprising the two subunits gp91^{phox} and p22^{phox}, that transfers electrons upon activation from NADPH to oxygen molecule. When cells are stimulated, the three cytosolic proteins p47^{phox}, p67^{phox}, and the small GTPase Rac, each indispensable for the oxidase activation, translocate to the membrane where they assemble with the cytochrome.

p47^{phox} harbors two SH3 domains, which specifically interact with the C-terminal cytoplasmic PRR of p22^{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^{phox}, a mutation that occurs in a patient with chronic granulomatous disease (5, 6, 23–25). Since the full-length wild-type p47^{phox} in resting phagocytes or the one expressed in *Escherichia coli* or budding yeast is incapable of binding to p22^{phox}, a resting form of p47^{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^{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^{phox}. Anionic amphiphiles such as arachidonate and SDS, activators of the oxidase *in vitro* (27), cause a conformational change of p47^{phox} to expose the SH3

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‖ To whom correspondence should be addressed. Tel.: 81 92-642-6213; Fax: 81 92-642-6215; E-mail: hsumi@mailserver.med.kyushu-u.ac.jp.

[1] The abbreviations used are: SH3, Src homology 3; PRR, proline-rich region; PMA, phorbol 12-myristate 13-acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; EBV, Epstein-Barr virus; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; GTPγS, 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 $p47^{phox}$ ($p47\text{-}\Delta C$; 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 $p22^{phox}$ and activating the oxidase in the absence of the amphiphile activators (26).

It is well established that, upon cell stimulation, $p47^{phox}$ 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 $p47^{phox}$ (Ser-303, Ser-304, Ser-315, Ser-320, Ser-328, Ser-345, Ser-348, Ser-359, Ser-370, and Ser-379) are phosphorylated when human neutrophils were stimulated with the protein kinase C activator PMA or the chemotactic formyl peptide fMLP. The phosphorylation is likely involved in activation of the phagocyte NADPH oxidase, because the mutant $p47^{phox}$ carrying the double substitution S303A/S304A only marginally corrects the defect in superoxide production in EBV-transformed $p47^{phox}$-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 $p47^{phox}$ and a conformational change that leads to the oxidase activation. As an initial step of the analyses, we replaced the serines of $p47^{phox}$ 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 $p22^{phox}$. Furthermore, the triply mutated $p47^{phox}$ 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 $p47^{phox}$ induces a conformational change to a state accessible to $p22^{phox}$, thereby activating the NADPH oxidase.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The DNA fragments encoding the full-length of $p47^{phox}$ (p47-F; amino acid residues 1–390), p47-(SH3)2-(154–286), p47-SH3(N)-(154–219), p47-SH3(C)-(223–286), $p47\text{-}\Delta C$-(1–286), p47-(1–302), p47-(1–314), p47-(1–327), and p47-(1–340) were amplified from a cloned cDNA encoding human $p47^{phox}$ 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 $p22^{phox}$, p22-C-(132–195) and its mutant p22-C-(P156Q), were prepared as described previously (5, 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 competent 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. 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 manufacturers' 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 Na_2HPO_4, and 1.47 mM KH_2PO_4) 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 amylose in 50 mM Tris-HCl (pH 8.0), respectively. The eluates were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

Cell-free Activation of the Phagocyte NADPH Oxidase—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 $p47^{phox}$ fused to GST, an N-terminal fragment of $p67^{phox}$ (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 7.0, containing 75 μM cytochrome *c*, 10 μM FAD, 1.0 mM EGTA, 1.0 mM $MgCl_2$, and 1.0 mM NaN_3. 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, pSXLC/pHa, that utilizes an internal ribosome entry site fragment of encephalomyocarditis virus (38) to transduce the $gp91^{phox}$ gene into the leukemia cell line K562 that expresses $p22^{phox}$ but not $gp91^{phox}$ (39). Cells highly expressing $gp91^{phox}$ were selected using FACS scan with the monoclonal antibody 7D5 to detect functional cytochrome b_{558} comprising the two subunits $gp91^{phox}$ and $p22^{phox}$ (40). A bicistronic retrovirus vector encoding a human multidrug resistance gene (*MDR1*) and the $p67^{phox}$ gene (pHa-MDR-IRES-p67) (41) was further transduced to the stably transduced $gp91^{phox}$-expressing K562 cells. The doubly transduced cells were selected with 4 ng/ml vincristine, expanded in a drug-free medium, and used for the following experiments.

Complementary DNAs encoding the full-length of the wild-type and mutant $p47^{phox}$ carrying the S328A or S303A/S304A substitution were subcloned into pREP4 (Invitrogen), which were transfected by electroporation to the K562 cells that stably express both $gp91^{phox}$ and $p67^{phox}$. The K562 cells (2×10^7 cells/ml) were electroporated in the presence of 10 μg of the wild-type or mutant form of $p47^{phox}$ plasmid DNA at 170 V, 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 $p47^{phox}$ and $p67^{phox}$, K562 cells (1×10^5 cells) were lysed by sonication, and the sonicates were applied to 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and probed with anti-$p47^{phox}$ and anti-$p67^{phox}$ 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 (1×10^5 cells) expressing the wild-type or mutant $p47^{phox}$ 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 $p47^{phox}$ Is Capable of Binding to $p22^{phox}$—The two SH3 domains of $p47^{phox}$ are tandemly arranged in the central portion in the primary sequence: the N-terminal one of 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 $p47^{phox}$ (amino acids 286–390) interacts with the SH3 domains in an intramolecular fashion, which likely prevents the domains from binding to $p22^{phox}$ (5, 26). To map precisely the region responsible for the prevention, we expressed and purified the full-length $p47^{phox}$ (p47-F; amino acids 1–390) and a series of C-terminally truncated $p47^{phox}$ (p47-(1–340), p47-(1–327), p47-(1–314), p47-(1–302), and p47-ΔC-(1–286)) as GST fusion proteins (Fig. 2*A*), and we tested their ability to bind to the C-terminal cytoplasmic tail of $p22^{phox}$ (amino acids 132–195), p22-C. As shown in Fig. 2*B*, GST-p47-ΔC-(1–286) was fully precipitated with amylose resin coupled to MBP-p22-C, whereas GST alone was hardly recovered. The interaction seems to be mediated via the SH3 domains of $p47^{phox}$, since $p47\text{-}\Delta C$ 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. 2*B*). On the other hand, neither p47-(1–314) nor $p47^{phox}$ 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. 2*A*), 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 $p47\text{-}\Delta C$ or with p47-(1–302) (data not shown). These results were confirmed by an *in vivo* 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 $p47\text{-}\Delta C$ or p47-(1–302) (data not shown).

The Region of Amino Acids 286–340 in $p47^{phox}$ 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 $p47^{phox}$ and $p22^{phox}$, probably by intramolecularly binding to the SH3 domains. To test this possibility, we expressed a various length of C-terminal fragments of $p47^{phox}$, namely p47-(286–302), p47(286–314), p47-(286–327), p47-(286–340), and p47-C-(286–390), as MBP fusions (Fig. 3*A*), and we tested their ability to bind to the SH3 domains of $p47^{phox}$, p47-(SH3)2. As shown in Fig. 3*B*, p47-(SH3)2 directly but weakly interacted with p47-(286–314), whereas it failed to bind to p47-(286–302) or MBP alone. A slightly stronger interaction was observed with p47-(286–327). A longer fragment, p47-(286–340), bound to p47-(SH3)2 to the same extent as the full-length C-terminal peptide p47-C-(286–390) did. On the other hand, the C-terminal tail of $p47^{phox}$ (amino acids 360–390), which contains a proline-rich region (amino acids 360–369; KPQPAVPPRP), did not interact with p47-(SH3)2 (data not shown), indicating that the extreme C terminus is not involved in the interaction. Thus the fragment of amino acids 286–314 likely conforms the minimally essential site for the SH3 domains, and its C-terminal flanking region (amino acids 315–340) is required for the stable intramolecular interaction. This region (amino acids 286–340) is highly conserved between human and mouse $p47^{phox}$ with 84% identity and 96% similarity (42). In contrast, there exists much less identity (26%) in the region of amino acids 341–359 (42).

The Tandem SH3 Domains Synergistically Interact with the Region of Amino Acids 286–340 in $p47^{phox}$—To clarify the role for each of the two SH3 domains of $p47^{phox}$ in the intramolecular interaction, we compared the ability to bind to the target region of amino acids 286–340 among p47-(SH3)2, p47-SH3(N), and p47-SH3(C). As shown in Fig. 4*A*, a negligible binding activity was observed when p47-SH3(N) or p47-SH3(C) was used instead of p47-(SH3)2. Thus the tandem SH3 domains appear to interact synergistically with the intramolecular target site to keep $p47^{phox}$ in a closed inactive conformation.

The Stretch PPRR of Amino Acids 299–302 Plays an Essential Role in the Intramolecular Interaction of $p47^{phox}$—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 RXΦPXΦP (type I ligand) and/or ΦPXΦPXR (type II ligand), and thus PXΦP is generally accepted as the target motif of SH3 domains accordingly (43–46). Such a PXΦP motif, however, is absent in the intramolecular SH3 target region of $p47^{phox}$ (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), appears to be a remnant of the type II

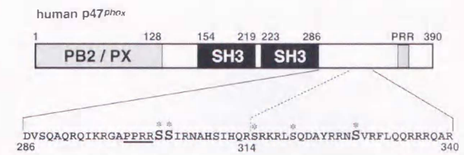

FIG. 1. **Structure of human $p47^{phox}$.** The structure of human $p47^{phox}$ is schematically represented. The two SH3 domains are tandemly arranged in the central portion in the primary sequence as follows: the N-terminal one of amino acid residues 154–219 and the C-terminal one of residues 223–286. The amino acid sequence is shown in the intramolecular SH3 target region (amino acids 286–340), where the PPRR stretch (amino acids 299–302) is *underlined* and serines with *asterisks* are the ones that become phosphorylated upon cell stimulation. *Gray* boxes represent the PB2/PX domain (amino acids 1–128) (Ref. 22) and the PRR (amino acids 360–369).

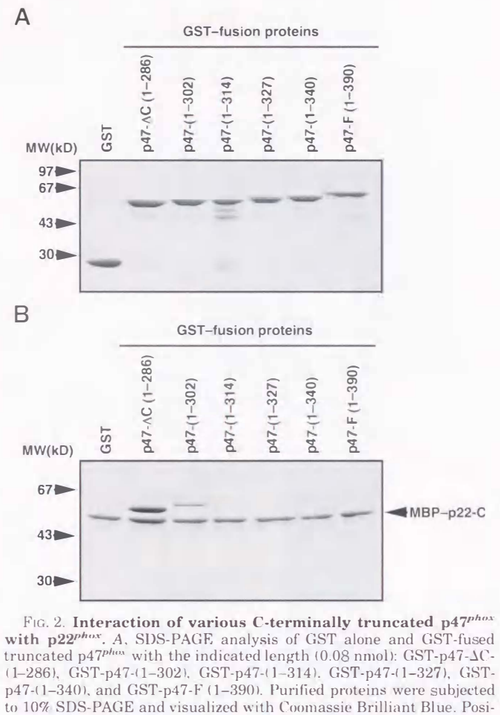

FIG. 2. **Interaction of various C-terminally truncated $p47^{phox}$ with $p22^{phox}$.** *A*, SDS-PAGE analysis of GST alone and GST-fused truncated $p47^{phox}$ with the indicated length (0.08 nmol): GST-$p47\text{-}\Delta C$-(1–286), GST-p47-(1–302), GST-p47-(1–314), GST-p47-(1–327), GST-p47-(1–340), and GST-p47-F (1–390). Purified proteins were subjected to 10% SDS-PAGE and visualized with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa. *B*, *in vitro* pull-down binding assay. GST alone or the indicated GST-fused $p47^{phox}$ (0.15 nmol) was incubated with 0.15 nmol of MBP-p22-C (amino acids 132–195) and pulled down with amylose resin. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa. For details, see "Experimental Procedures."

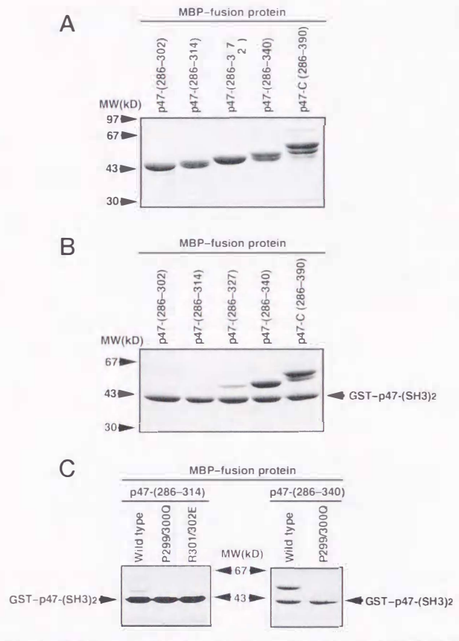

FIG. 3. **Mapping of the region responsible for an SH3 domain-mediated intramolecular interaction in p47^{phox}.** *A*, SDS-PAGE analysis of MBP-fused C-terminal fragments of p47^{phox} (0.1 nmol): MBP-p47-(286–302), MBP-p47-(286–314), MBP-p47-(286–327), MBP-p47-(286–340), and MBP-p47-C (286–390). Purified proteins were subjected to 10% SDS-PAGE and visualized with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa. *B*, p47-(SH3)2 (amino acids 154–286) (0.15 nmol) was incubated with 0.3 nmol of the indicated MBP fusion protein and pulled down with glutathione-Sepharose 4B. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa. *C*, GST-p47-(SH3)2 (0.15 nmol) was incubated with 0.3 nmol of MBP-p47-(286–314) or the one carrying the P299Q/P300Q or R301/302E substitution (*left*), or with 0.3 nmol of MBP-p47-(286–340) or the one carrying the P299/300Q substitution (*right*), and pulled down with glutathione-Sepharose-4B. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa.

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 abrogated by these substitutions (Fig. 3*C*). The P299Q/P300Q substitution also resulted in a loss of a stronger interaction between p47-(286–340) and the SH3 domains (Fig. 3*C*). Thus the PPRR stretch of 299–302 likely conforms the core-binding site for the SH3 domains in the intramolecular interaction.

A proline residue in an SH3-targeted peptide directly contacts a conserved tryptophan residue in the binding surface of the SH3 domain (43–46). To know which SH3 domain of p47^{phox} acts as the partner for the PPRR stretch, we introduced a substitution of arginine for either of the conserved tryptophans in the two SH3 domains as follows: p47-(SH3)2 (W193R) with the substitution in the N-terminal SH3 domain and p47-(SH3)2 (W263R) with that in the C-terminal one. The W193R substitution abolished the interaction with p47-(286–340), whereas the replacement of Trp-263 led to only a partial defect

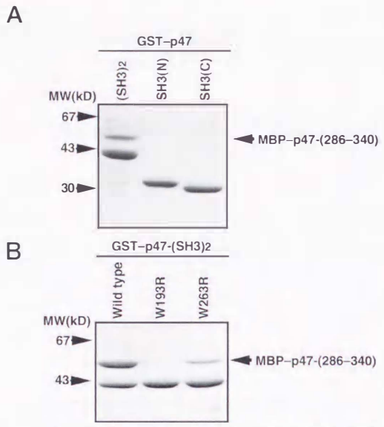

FIG. 4. **Role for the two SH3 domains of p47^{phox} in the intramolecular interaction.** *A*, GST-p47-(SH3)2, GST-p47-SH3(N), or GST-p47-SH3(C) (0.15 nmol) was incubated with 0.2 nmol of MBP-p47-(286–340), and pulled down with glutathione-Sepharose 4B. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa. *B*, GST-p47-(SH3)2, GST-p47-(SH3)2 (W193R), or GST-p47-(SH3)2 (W263R) (0.15 nmol) was incubated with 0.3 nmol of MBP-p47-(286–340) and pulled down with glutathione-Sepharose 4B. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa.

(Fig. 4*B*). The finding indicates that the intramolecular interaction requires, probably as the partner for the PPRR stretch, the Trp-193-centered binding surface of SH3(N), which also participates in the intermolecular interaction with p22^{phox} (24).

Mutations in the PPRR Stretch Render p47^{phox} in a Conformation Capable of Binding to p22^{phox}—The dual role for the N-terminal SH3 domain of p47^{phox}, *i.e.* its involvement in both intra- and intermolecular interactions via the same binding surface, suggests that disruption of the intramolecular interaction in p47^{phox} directly promotes the accessibility of the SH3 domain to p22^{phox}. To investigate this possibility, we expressed the full-length mutant protein of p47^{phox} carrying the P299Q/P300Q or R301E/R302E substitution, in each of which the intramolecular interaction is expected to be disrupted. As shown in Fig. 5*A*, 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. 5*B*); MBP-p22-C directly interacted with the mutated proteins but not with the wild-type one (Fig. 5*C*). Taken together, we concluded that p47^{phox} becomes accessible 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, Ser-315, Ser-320, 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,

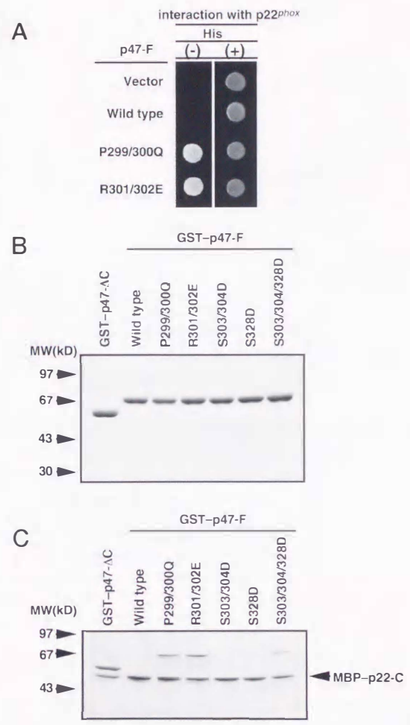

FIG. 5. **Effect for amino acid replacement in p47^{phox} on the interaction with p22^{phox} both *in vivo* and *in vitro*.** *A*, the yeast Y190 cells were co-transformed with both pGBT9-p22-C and pACT2-p47-F, pACT2-p47-F (P299Q/P300Q), or pACT2-p47-F (R301E/R302E). Following the selection for Trp$^+$ and Leu$^+$ phenotype, its histidine-dependent (*right*) and -independent (*left*) growth was tested as described under "Experimental Procedures." *B*, SDS-PAGE analysis of various mutants of p47^{phox} (0.1 nmol): GST-p47-ΔC-(1–286), GST-p47-F, GST-p47-F (P299Q/P300Q), GST-p47-F (R301E/R302E), GST-p47-F (S303D/S304D), GST-p47-F (S328D), and GST-p47-F (S303D/S304D/S328D). Purified proteins were subjected to 10% SDS-PAGE and visualized with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa. *C*, MBP-p22-C (0.15 nmol) was incubated with 0.15 nmol of GST-p47-ΔC-(1–286), GST-p47-F, GST-p47-F (P299Q/P300Q), GST-p47-F (R301E/R302E), GST-p47-F (S303D/S304D), GST-p47-F (S328D), or GST-p47-F (S303D/S304D/S328D), and pulled down with amylose resin. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa.

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 p47^{phox} 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. 6*A*), raising the possibility that additional modification may be required for conversion of p47^{phox} to a state accessible to p22^{phox}.

To identify other serine residues to be modified, we truncated p47^{phox} (S303D/S304D) from the C terminus. The two-hybrid experiment revealed that p47-(1–327, S303D/S304D), but not p47-(1–340, S303D/S304D), was capable of interacting with p22-C (Fig. 6*A*). Since Ser-328 is the only serine to be phosphorylated upon cell stimulation within the region of 328–340 and, in addition, becomes phosphorylated as intensively as Ser-303

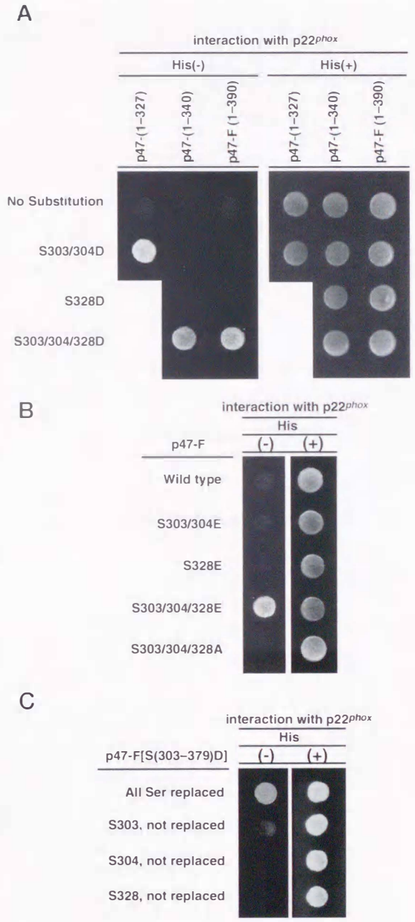

FIG. 6. **Effect for replacement of serines in p47^{phox} on the interaction with p22^{phox}.** *A*, the yeast Y190 cells were co-transformed with both pGBT9-p22-C and pACT2 encoding p47-F, p47-(1–340), or p47-(1–327) carrying the S303D/S304D, S328D, or S303D/S304D/S328D substitution. Following the selection for Trp$^+$ and Leu$^+$ phenotype, its histidine-dependent (*right*) and -independent (*left*) growth was tested as described under "Experimental Procedures." *B*, the yeast Y190 cells were co-transformed with pGBT9-p22-C and pACT2 encoding the full-length p47^{phox} carrying the S303E/S304E, S328E, S303E/S304E/S328E, or S303A/S304A/S328A substitution. Following the selection for Trp$^+$ and Leu$^+$ phenotype, its histidine-dependent (*right*) and -independent (*left*) growth was tested as described under "Experimental Procedures." *C*, in p47-F(S(303–379)D), 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). The yeast Y190 cells were co-transformed with pGBT9-p22-C and pACT2 encoding p47-F(S(303–379)D) or the one carrying substitution for all but one serine among Ser-303, Ser-304, or Ser-328. Following the selection for Trp$^+$ and Leu$^+$ phenotype, its histidine-dependent (*right*) and -independent (*left*) growth was tested as described under "Experimental Procedures."

and Ser-304 (32), we introduced the additional substitution S328D to obtain p47-F (S303D/S304D/S328D). As shown in Fig. 6*A*, p47-(1–340) and the full-length p47^{phox} carrying the triple substitution did interact with p22-C, whereas those carrying

TABLE I
Interaction between full-length p47^{phox} carrying replacement of serines and p22^{phox} in the yeast two-hybrid system

Histidine-independent growth was tested using yeast Y190 cells that were co-transformed by pairs of pGBT9 encoding p22^{phox} and pACT encoding full-length p47^{phox} carrying replacement of serines as described under "Experimental Procedures."

p47^{phox}	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/S328D	+
S303D/S304D/S320D/S328D	+
S303D/S304D/S315D/S320D/S328D	+
S345D/S348D	−
S303D/S304D/S345D/S348D	−
S328D/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. 6*B*). On the other hand, alanines could not replace aspartates or glutamates (Fig. 6*B*).

All the three serines (Ser-303, Ser-304, and Ser-328) to be replaced are likely required for p47^{phox} to interact with p22^{phox}, 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 (S303/304D) 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^{phox}.

Triple Replacement of Ser-303, Ser-304, and Ser-328 Is Required for p47^{phox} to Interact with p22^{phox}—To clarify the effect of each substitution of aspartate for Ser-303, Ser-304, or Ser-328, we constructed a mutant p47^{phox}, designated p47-F (S(303–379)D), 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. 6*C*, the protein p47-F (S(303–379)D) bound 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^{phox} to interact with p22^{phox}.

Triple Replacement of Ser-303, Ser-304, and Ser-328 Results in Disruption of the SH3-mediated Intramolecular Interaction in p47^{phox}—It seems likely that the binding of the triply mutated p47^{phox} (S303D/S304D/S328D) to p22^{phox} results 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-(SH3)2. The fragment carrying the S303D/S304D or S328D substitution bound to p47-(SH3)2 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^{phox} gains access to p22^{phox} (Figs. 5*C* and 6*A*). Taken together, simultaneous phosphorylation of Ser-303, Ser-304, and Ser-328 of p47^{phox} appears to primarily disrupt the SH3-mediated intramolecular interaction, thereby leading to the interaction of the unmasked SH3 domain with p22^{phox}.

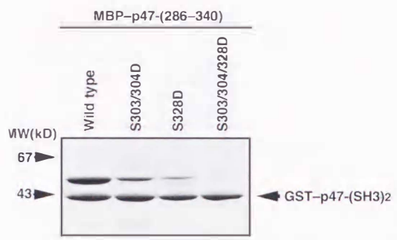

FIG. 7. **Effect of replacement of Ser-303, Ser-304, and Ser-328 with aspartate on the interaction of the SH3 domains with their target region of p47^{phox}.** GST-p47-(SH3)2 (0.15 nmol) was incubated with 0.3 nmol of MBP-p47-(286–340) or the one carrying the S303D/S304D, S328D, or S303D/S304D/S328D substitution and pulled down with glutathione-Sepharose 4B. The precipitated proteins were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Positions for marker proteins are indicated in kDa.

Mutant p47^{phox} Proteins That Are Accessible to p22^{phox} 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^{phox} 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^{phox}. 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 b_{558} and three cytosolic proteins: p47^{phox}, p67^{phox}, 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 vitro* by p47^{phox} and p67^{phox}, both in C-terminally truncated forms, in the presence of the GTP-bound Rac (26). When the full-length p47^{phox} (p47-F) is used instead of the truncated p47^{phox}, p47-ΔC (residues 1–286), the activation absolutely requires the amphiphiles (Ref. 26 and Fig. 8). The finding implies that p47^{phox} is a target of the amphiphiles and that p47-ΔC serves as an active form of p47^{phox}.

By using this system, we tested whether mutant p47-F proteins mimicking a phosphorylated form can replace p47-ΔC to activate the oxidase *in vitro* without the amphiphiles. As shown in Fig. 8*A*, 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,

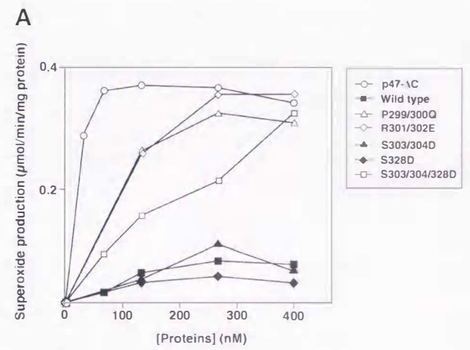

FIG. 8. **Activity of mutant p47^{phox} to support superoxide production in an anionic amphiphile-independent cell-free system for the NADPH oxidase activation.** *A*, human neutrophil NADPH oxidase was activated with the indicated concentration of the wild-type or mutant GST-p47^{phox}, GST-p67^{phox}-N (amino acids 1–242) (10 μg/ml), His-tagged Rac2 (10 μg/ml) preloaded with 100 μM GTPγS, and human neutrophil membranes (10 μg/ml), in the absence of the anionic amphiphile activators. Superoxide production was determined as described under "Experimental Procedures." *Open circle*, p47-ΔC-(1–286); *closed square*, the wild-type p47-F; *open triangle*, p47-F (P299Q/P300Q); *open diamond*, p47-F (R301E/R302E); *closed triangle*, p47-F (S303D/S304D); *closed diamond*, p47-F (S328D); and *open square*, p47-F (S303D/S304D/S328D). *B*, human neutrophil NADPH oxidase was activated with the indicated p47^{phox} protein (100 nM) under the conditions as described in *A*, except that SDS (100 μM) was present.

compared with p47-ΔC. Both p47-F (S303E/S304E/S328E) and p47-F (S(303–379)D) were as 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. 5*C*), was incapable of activating the oxidase without amphiphiles (Fig. 8*A*), although these mutant proteins are as active as the wild-type p47^{phox} in the presence of the amphiphile activator SDS (Fig. 8*B*). 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. 8*A*, these proteins could activate the oxidase in an amphiphile-independent manner. The order of the potency to activate the oxidase (Fig. 8*A*) is identical with that to bind to p22^{phox} (Fig. 5*C*): p47-ΔC > p47-F (P299Q/P300Q) = p47-F (R301E/R302E) > p47-F (S303D/S304D/S328D) ≫ p47-F (S328D) = p47-F (S303D/S304D). Thus the binding of p47^{phox} 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. 9*A*).

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. 9*A*). 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

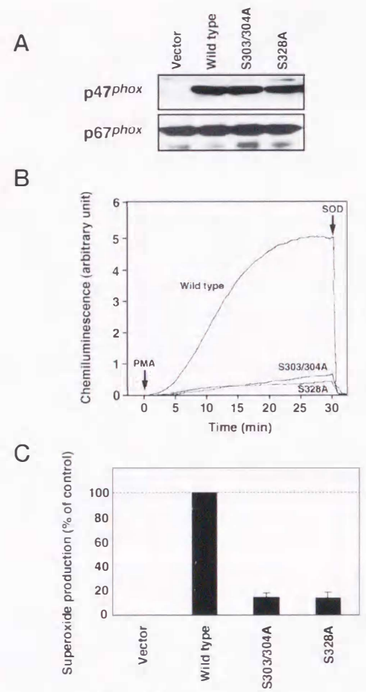

FIG. 9. **Effect of the S328A or S303A/S304A substitution in $p47^{phox}$ on the NADPH oxidase activation in a whole-cell system.** *A*, expression of $p47^{phox}$ in $gp91^{phox}$ and $p67^{phox}$-transduced K562 cells. The doubly transduced K562 cells were transfected with the pREP4 vector alone or pREP4 encoding the wild-type or mutant $p47^{phox}$ carrying the substitution S303A/S304A or S328A. In the *upper panel*, cell lysates were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with an anti-$p47^{phox}$ monoclonal antibody. In the *lower panel*, cell lysates were immunoblotted with an anti-$p67^{phox}$ monoclonal antibody. *B*, PMA-induced chemiluminescence by $gp91^{phox}$ and $p67^{phox}$-transduced K562 cells transfected with pREP4 encoding the wild-type or mutant $p47^{phox}$ carrying the substitution S303A/S304A or S328A. The K562 cells expressing the indicated form of $p47^{phox}$ (1×10^5 cells) were stimulated with PMA (200 ng/ml), and chemiluminescence change was continuously monitored with an enhanced luminol-based substrate, DIOGENES. Superoxide dismutase (*SOD*) (50 μg/ml) was added where indicated. *C*, relative superoxide-producing activities in PMA-stimulated K562 cells transfected with the wild-type or mutant of $p47^{phox}$. Superoxide production is expressed as the percent activity relative to control cells transfected with the wild-type $p47^{phox}$. Each graph represents the mean of data from three independent transfections, with *bars* representing the standard deviation of percent activity.

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. 3*B*). The SH3-binding site, somewhat to our surprise, lacks the canonical SH3 target motif PXΦP; 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. 3*C*). In addition, full-length mutant proteins of $p47^{phox}$, P299Q/P300Q and R301E/R302E, are capable of not only binding to the intermolecular SH3 target $p22^{phox}$ (Fig. 5, *A* and *C*) but also activating the NADPH oxidase under cell-free conditions without the amphiphile activators (Fig. 8*A*). These observations establish that the SH3-mediated intramolecular interaction is the major determinant for keeping $p47^{phox}$ in a closed inactive conformation.

The interaction of $p47^{phox}$ with $p22^{phox}$ appears to be a rate-limiting step in the oxidase activation, as indicated by the observation that the $p22^{phox}$ binding activity of various mutant $p47^{phox}$ is completely parallel with their ability to activate the oxidase (Fig. 8*A*). Thus a conformation capable of engaging $p22^{phox}$, resulting from disruption of the SH3-mediated intramolecular interaction, represents an active state of $p47^{phox}$.

Reason for the Two SH3 Domains to Be Tandemly Arrayed in $p47^{phox}$: For the Refined Regulation of the Conformational Change?—The intramolecular interaction that determines active and inactive conformation of $p47^{phox}$ requires both SH3 domains. The N-terminal SH3 domain of $p47^{phox}$ (amino acids 154–219) appears to contact directly with the PPRR 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. 4*B*). 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 $p47^{phox}$. 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. 4*A*). 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 $p47^{phox}$, 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 $p47^{phox}$.

Triple Replacement of Ser-303, Ser-304, and Ser-328 in $p47^{phox}$ with Aspartates, a Mutation That Is Sufficient for Disruption of the SH3-mediated Intramolecular Interaction, Induction of Binding to $p22^{phox}$, and Activation of the NADPH Oxidase—It is well established that stimulation of human neutrophils leads to extensive phosphorylation of $p47^{phox}$ 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 $p47^{phox}$ 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 $p22^{phox}$. 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 $p47^{phox}$ 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 amphiphile activators (Fig. 8*A*). On the other hand, mutant $p47^{phox}$ 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 $p47^{phox}$ likely functions as a switch from a closed inactive conformation to a state capable of both binding to $p22^{phox}$ 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 $p47^{phox}$.

Role for Phosphorylation of Ser-328 as Well as Ser-303 and Ser-304 in $p47^{phox}$—A recent study has suggested the importance of phosphorylation at serines 303 and 304 in the oxidase activation; the double mutant $p47^{phox}$ S303A/S304A is much less active than the wild-type one when expressed in EBV-transformed $p47^{phox}$-deficient B cells (33). It has remained, however, unknown what is induced in $p47^{phox}$ 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 $p47^{phox}$, since the S328A substitution results in defective activation of the oxidase *in vivo* (Fig. 9). The strict requirement for the three serines to be phosphorylated is also suggested by the finding that a protein without substitution of aspartate for any of Ser-303, Ser-304, or Ser-328 is incapable of interacting with $p22^{phox}$, even when all other serines to be phosphorylated are replaced with aspartates (Fig. 6*C*).

Role for Phosphorylation of Other Serines in $p47^{phox}$—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 $p47^{phox}$ carrying the S359D/S370D/S379D substitution fails to interact with $p22^{phox}$ (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 $p22^{phox}$ (Figs. 5 and 6 and Table I). Serines 359 and 370 are located just N- and C-terminally to the PRR of $p47^{phox}$ (amino acids 360–369; KPQPAVPPRP), respectively. The PRR is constitutively occupied by the C-terminal SH3 domain of $p67^{phox}$, a binding that occurs in a manner independent of the SH3-mediated intramolecular interaction in $p47^{phox}$ (26). Thus both Ser-359 and Ser-370 are likely sequestered from the intramolecularly interacting moiety of $p47^{phox}$, 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 $p47^{phox}$—Based on the present findings, here we propose a model that phosphorylation of $p47^{phox}$ induces a conformational change to a state accessible to $p22^{phox}$, thereby activating the NADPH oxidase (Fig. 10). In a resting state, $p47^{phox}$ 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. The disruption renders $p47^{phox}$ in an open conformation capable of interacting with $p22^{phox}$ via the unmasked SH3 domains. The induced interaction between the oxidase factors serves as a rate-limiting step to activate the NADPH oxidase. Since at least one tryptophan residue, Trp-193, plays a critical role in keeping $p47^{phox}$ in the SH3-mediated closed conformation, this model likely explains an observation by Quinn's group (28) that phosphorylation of $p47^{phox}$ by protein kinase C *in vitro* results in quenching in the intrinsic tryptophan fluorescence, which correlates well with NADPH oxidase activity.

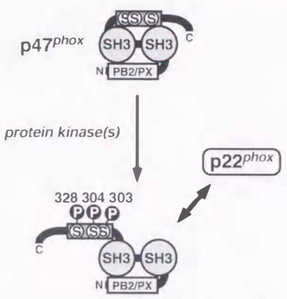

FIG. 10. **A model for phosphorylation-dependent activation of $p47^{phox}$.** In a resting state, $p47^{phox}$ is folded in a closed inactive conformation by the intramolecular interactions via the two SH3 domains. Simultaneous phosphorylation of Ser-303, Ser-304, and Ser-328 disrupts the intramolecular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

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