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Cterminally Truncated Forms IMPLICATION FOR
REGULATORY SH3 DOMAIN-MEDIATED INTERACTIONS

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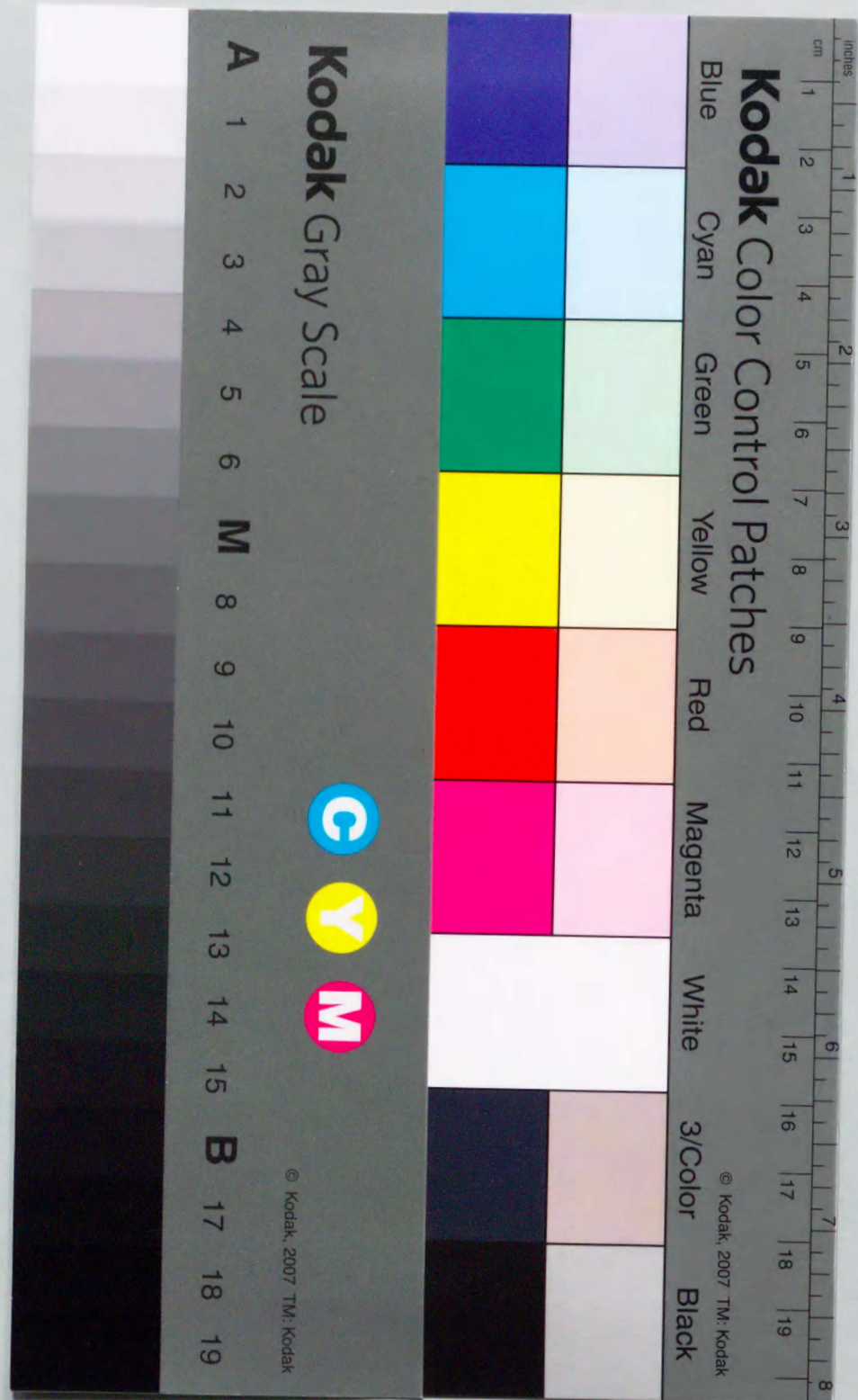
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IMPLICATION FOR REGULATORY SH3 DOMAIN-MEDIATED INTERACTIONS*

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Anionic amphiphiles, such as arachidonate, activate the superoxide-producing phagocyte NADPH oxidase in a cell-free system with human neutrophil membrane, which contains cytochrome *b*₅₅₈ comprising gp91^{phox} and p22^{phox}, and three cytosolic proteins: p47^{phox} and p67^{phox}, each harboring two SH3 domains, and the small GTPase Rac. Here we show that, even without the amphiphiles, the oxidase is activated *in vitro* by a C terminally truncated p47^{phox}, retaining the N-terminal and the two SH3 domains, and the N terminus of p67^{phox}. When either truncated p47^{phox} or p67^{phox} is replaced by the respective full-length one, the activation absolutely requires the amphiphiles. The results indicate that both p47^{phox} and p67^{phox} are the primary targets of the amphiphiles, and that their C-terminal regions play negative regulatory roles. We also find that the truncated p47^{phox}, but not the full-length one, can bind to p22^{phox}, a binding required for the oxidase activation. The N-terminal SH3 domain of p47^{phox} is responsible for the binding not only to p22^{phox}, but also to the p47^{phox} C terminus. Thus the SH3 domain is accessible in the active p47^{phox}, but is normally masked in the full-length one probably via intramolecularly interacting with the C terminus. The present findings support our previous proposal of regulatory SH3 domain-mediated interactions.

Increasing attention has currently been paid to specific protein-protein interactions in intracellular signal transduction, which are mediated by modular binding domains of signaling proteins (1, 2). Among the domains to be characterized at an earlier stage is the Src homology 3 (SH3)¹ domain found in various proteins including the Src family tyrosine kinases. The domain directly binds, via its target-binding surface, to a proline-rich region (PRR) of its partners, thereby mediating protein-protein interactions (1–4). Unlike the case of SH2 do-

mains, whose interactions with tyrosine-containing peptides are promoted by phosphorylation of the SH2 domain-binding site, the regulatory mechanism for SH3 domain-mediated associations largely remains elusive.

Specific interactions via SH3 domains play a crucial role in assembly and activation of the phagocyte NADPH oxidase (5–15). The oxidase, dormant in resting cells, is activated during phagocytosis to catalyze reduction of molecular oxygen to superoxide, a precursor of microbicidal oxidants (16–20). The significance of this enzyme in host defense is made evident by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease, whose phagocytes lack the superoxide-producing system (16–20). Recent studies, furthermore, have suggested that oxidants produced by the NADPH oxidase may be also involved in Ras-mediated mitogenic signaling in fibroblasts (21), oxygen sensing in airway chemoreceptors (22), and activation of c-Jun N-terminal kinase in kidney epithelial cells (23). The catalytic core of the oxidase, which transfers electrons from NADPH to oxygen molecule, is the membrane-integrated flavocytochrome *b*₅₅₈, composed of the two subunit gp91^{phox} and p22^{phox} (16–20). Activation of the oxidase requires translocation of three cytosolic proteins, p47^{phox}, p67^{phox}, and the small GTPase Rac1/2, to the membrane where they assemble with the cytochrome. Both p47^{phox} and p67^{phox} harbor two SH3 domains, which mediate specific interactions between the oxidase factors. The C-terminal SH3 domain of p67^{phox} interacts with the PRR of p47^{phox} (6, 8, 12), while the N-terminal one of p47^{phox} does with p22^{phox} (11, 15). The latter interaction is required for both translocation of p47^{phox} and activation of the NADPH oxidase (5, 8–11, 15). A monoclonal antibody specific for the p47^{phox} SH3 domains interacts with p47^{phox} in the presence of arachidonic acid, an activator of the oxidase, but not with the resting form of p47^{phox} (5). It is likely that the N-terminal SH3 domain of p47^{phox} is normally inaccessible, and, upon activation, becomes unmasked to interact with p22^{phox} (5, 11). In the oxidase system, thus, the SH3 domain-mediated interactions are apparently regulated in contrast with the Grb2/Sos SH3 domain-mediated contacts, which are constitutive (1, 2).

In addition to the whole cell activation by various phagocytic or non-phagocytic stimuli, the NADPH oxidase can be activated in a cell-free system reconstituted with cytochrome *b*₅₅₈ and the three cytosolic proteins, p47^{phox}, p67^{phox}, and Rac in the GTP-bound form (17–20). In the system, the activation is totally dependent on the addition of such anionic amphiphile activators as arachidonic acid and sodium dodecyl sulfate (SDS) (24). Here we have developed an *in vitro* system in which the NADPH oxidase is activated without using the amphiphile

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¹ The abbreviations used are: SH3, Src homology 3; PRR, proline-rich region; GST, glutathione *S*-transferase; GTPγS, guanosine 5'-3-*O*-(thio)-triphosphate; GDPβS, guanosine 5'-2-*O*-(thio)diphosphate; PAGE, polyacrylamide gel electrophoresis.

activators. A mutant p47^{phox}, allowing the N-terminal SH3 domain unmasked, is capable of both binding to p22^{phox} and activating the oxidase without the amphiphiles when used with the N terminus of p67^{phox}. These findings indicate the regulatory intramolecular association of the SH3 domain in p47^{phox}, which is directly linked to the oxidase activation.

EXPERIMENTAL PROCEDURES

Glutathione S-Transferase (GST) Fusion Proteins—The DNA fragments encoding the full-length p47^{phox} (p47-F; amino acid residues 1–390), p47-N (1–153), p47-(SH3)₂ (154–286), p47-F(W193R) (the full-length p47^{phox} with a substitution of Trp¹⁹³ for Arg), the full-length p67^{phox} (p67-F, 1–526), p67-N (1–242), p67-SH3(C) (455–526), and p22-C (the cytoplasmic domain of p22^{phox}, residues 132–195) were obtained as described previously (5, 11, 12). The DNAs for the p47-ΔC (1–286) and p47-ΔN (154–390) were amplified by polymerase chain reaction from a cloned cDNA encoding human p47^{phox} or p67^{phox}. For the mutant p47-F carrying the Trp²⁶³ → Arg substitution, namely p47-F(W263R), the mutation was introduced into p47-F by polymerase chain reaction-mediated site-directed mutagenesis. All the polymerase chain reaction products were subcloned into the pGEX-2T expression vector (Pharmacia Biotech, Uppsala, Sweden). All the plasmids were subjected to DNA sequencing for the confirmation of precise construction. The GST fusion proteins were expressed in *Escherichia coli* and purified by glutathione-Sepharose-4B beads (Pharmacia Biotech).

Cell-free Activation of the NADPH Oxidase—Both membrane and cytosolic fractions of human neutrophils were prepared by sequential centrifugations as described previously (5). To prepare Rac2-enriched fractions, the cytosolic fraction was applied to a 2',5'-ADP Sepharose CL-6B column. The flow-through fraction was applied to a DEAE Sepharose CL-6B column, and the Rac2-enriched fraction was eluted with 0.2 M NaCl. The fraction contained Rac2 but was free of p47^{phox} and p67^{phox} as confirmed by immunodetection (11).

The assay mixture was composed of 100 mM potassium phosphate (pH 7.0), 75 μM cytochrome c, 10 μM FAD, 10 μM GTPγS, 1.0 mM EGTA, and 1.0 mM Na₂S₂O₈. The neutrophil membrane was incubated for 2 min at room temperature with recombinant p47^{phox}, recombinant p67^{phox}, and the Rac2-enriched fraction in the presence or absence of 100 μM SDS. The reaction was then initiated by the addition of NADPH (1.0 mM) to the reaction mixture. The NADPH-dependent superoxide producing activity was measured by determining the rate of superoxide dismutase-inhibitable ferricytochrome c reduction at 550–540 nm with a dual-wavelength spectrophotometer (Hitachi 557), and was expressed as micromole/min/mg of membrane proteins (11).

Far Western Blotting—*In vitro* interaction between p47^{phox} and p22^{phox} was estimated by far Western blot as described previously (11). Briefly, the GST-p22-C (1.0 μg) was subjected to SDS-PAGE and transferred to nitrocellulose membrane, which was incubated with 10 μg of GST-p47-F, GST-p47-ΔC, or GST-p47-(SH3)₂ in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% bovine serum albumin. The filter was then probed with an anti-GST monoclonal antibody (25), a generous gift from Drs. Yoichi Tachibana (Nippon Zeon Corp., Tokyo, Japan) and Michiyuki Matsuda (International Medical Center of Japan, Tokyo, Japan), rather than a polyclonal anti-p47^{phox} antibody, because the latter may block the interaction with p22^{phox}. The monoclonal anti-GST antibody did not recognize the GST fusion protein transferred to nitrocellulose membrane after SDS-PAGE under the condition used (25). Complexes were detected using alkaline phosphatase-conjugated anti-mouse IgG antibodies.

Two-hybrid Experiments—The p47-F (amino acid residues 1–390), p47-ΔC (1–286), and the C-terminal region of p47^{phox}, namely p47-C (286–390), were cloned into a modified GAL4 activation domain-fusion vector pGAD424g (12) to obtain pGAD-p47-F, pGAD-p47-ΔC, and pGAD-p47-C, respectively. Deletion mutants of pGAD-p47-C, namely p47-ΔC1 and p47-ΔC2, lacked amino acid residues 299–346 and 360–390, respectively (12). The C-terminal SH3 domain of p67^{phox} (455–526), namely p67-SH3(C), and the C-terminal cytoplasmic tail of p22^{phox} (132–195), namely p22-C, were cloned into a modified GAL4 DNA-binding domain fusion vector pGBT9g (12) to obtain pGBT-p67-SH3(C) and pGBT-p22-C, respectively. All plasmids were subjected to DNA sequencing for the confirmation of precise construction.

Pairs between the pGAD and pGBT plasmids were cotransformed into competent yeast SFY526 cells with *HIS3* and *lacZ* reporter genes. After selection for Leu⁺ and Trp⁺ phenotypes, the transformants were tested for their ability to grow on plates lacking histidine. Activation of *lacZ* reporter was examined by β-galactosidase filter assay as described previously (12).

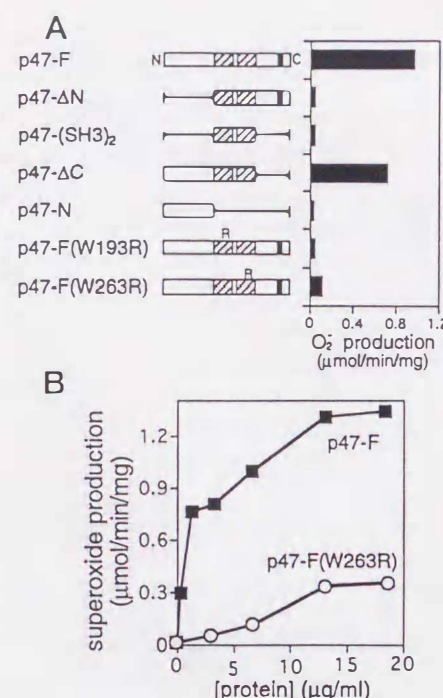


FIG. 1. Role of the domains of p47^{phox} in activation of the phagocyte NADPH oxidase *in vitro*. A, the full-length wild-type p47^{phox} (p47-F) or the indicated mutants were mixed with the neutrophil membrane (10.2 μg/ml), p67-F (2.1 μg/ml), and the Rac2-enriched fraction (35.8 μg/ml) in the presence of GTPγS (10 μM) and FAD (10 μM), followed by incubation with an optimal concentration of SDS (100 μM) for 2 min at room temperature. The reaction was initiated by the addition of NADPH (1.0 mM) to the reaction mixture, and the superoxide producing activity was determined as described under "Experimental Procedures." The values for the p47^{phox} mutants represent the activities at the concentration of 0.1 nmol/ml. The hatched and solid boxes represent the SH3 domains and the PRR of p47^{phox}, respectively. B, dose dependence of the *in vitro* oxidase activation on p47-F and p47-F(W263R).

RESULTS

Role of the p47^{phox} Regions in the NADPH Oxidase Activation in a Cell-free System—The protein p47^{phox}, an activating factor of the phagocyte NADPH oxidase, comprises four portions: the N-terminal region, the N-terminal and C-terminal SH3 domains, and the C-terminal tail (Fig. 1A). To investigate roles of the individual regions, we isolated deletion mutant proteins as GST fusions, and tested their abilities to activate the oxidase stimulated by SDS in a cell-free system reconstituted with human neutrophil membrane, the full-length p67^{phox}, and the Rac2-enriched fraction (Fig. 1A). In the cell-free system, the wild-type full-length p47^{phox} (p47-F) activated the NADPH oxidase in a dose-dependent manner (Fig. 1B), and the maximal activity was obtained at the concentration of 13.0 μg/ml (about 0.2 nmol/ml). At the concentration of 0.1 nmol/ml, the p47^{phox} lacking the C terminus (p47-ΔC) fully activated the oxidase, while neither the N terminally deleted one (p47-ΔN) nor the one without both termini (p47-(SH3)₂) was capable of supporting superoxide production (Fig. 1A). The latter two proteins were completely inactive even at 4-fold higher concentrations (data not shown). Thus the N-terminal region is essential for the oxidase activation. To clarify the role of the C-terminal SH3 domain, we introduced the substitution of Arg for Trp²⁶³, the most conserved residue in SH3 domains that directly interacts with a proline of target peptides (1, 2, 4). The mutation resulted in decreased ability to support superoxide production at submaximal (Fig. 1A) and saturated (Fig. 1B) concentrations, indicating that the SH3 domain is not essential, but is required for the full activation. This is in contrast

with that the N-terminal SH3 domain was an absolute requirement for the oxidase activation (Fig. 1A), which agrees with previous results by us and others (11, 15). Taken together, the N-terminal region and both SH3 domains are required for fully activating the oxidase.

The N-terminal Region of p67^{phox} Is Sufficient for the NADPH Oxidase Activation—Another oxidase activating protein p67^{phox} also harbors two SH3 domains (Fig. 2), both of which seem indispensable for superoxide production in stimulated cells (7). However, the domains and the region between them are not required in a cell-free system: the N-terminal region of p67^{phox} is sufficient for the oxidase activation (7), which was confirmed in our cell-free activation system (Fig. 2). The region (amino acid residues 1–242) contains the Rac-binding site (26), and the GTP-dependent interaction between p67^{phox} and Rac seems essential for the oxidase activation (26, 27). The activation accomplished by the C terminally deleted p47^{phox} (p47-ΔC) and the N terminus of p67^{phox} (p67-N) was essentially the same as that by their respective full-length proteins, in both the maximal activity (Fig. 2) and the dose dependence on the proteins (data not shown). Among the four portions of p67^{phox}, the N-terminal region is thus sufficient for the cell-free oxidase activation, in combination with the C terminally deleted p47^{phox} (p47-ΔC) as well as with full-length one (p47-F).

Anionic Amphiphile-independent Activation of the NADPH Oxidase in a Cell-free system—In the cell-free system with p47-F and p67-F, the oxidase activation was totally dependent on the anionic amphiphile SDS (Fig. 3A). On the other hand, to

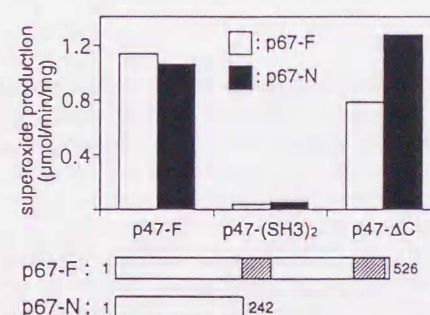


FIG. 2. Role of the N-terminal region of p67^{phox} in activation of the NADPH oxidase *in vitro*. The full-length wild-type p67^{phox} (p67-F, 5.9 μg/ml) or p67-N (7.2 μg/ml) was incubated with p47-F (6.5 μg/ml), p47-(SH3)₂ (6.5 μg/ml), or p47-ΔC (7.3 μg/ml), in the presence of 100 μM SDS, in the reaction mixture containing the neutrophil membrane, Rac2, GTPγS, and FAD. The superoxide producing activity was determined as described under "Experimental Procedures." The hatched boxes represent the SH3 domains of p67^{phox}.

our surprise, the NADPH-dependent superoxide production was observed even in the absence of the amphiphile, when both p47-ΔC and p67-N were used instead of the full-length ones (Fig. 3A). The activation required not only the truncated proteins (Fig. 3B) but also the neutrophil membrane and Rac2, but was diminished in the presence of GDPβS, an agent keeping Rac2 in an inactive form (Fig. 3C). These properties confirm that the superoxide production is indeed catalyzed by the phagocyte NADPH oxidase.

In combination with the full-length p67^{phox} (p67-F), the C terminally deleted p47^{phox} (p47-ΔC) failed to activate the oxidase in the absence of the amphiphile activator (Fig. 4). Similarly, without the activator, combination of p47-F and p67-N did not lead to superoxide production (Fig. 4). These results indicate that the activator directly interacts with both p47^{phox} and p67^{phox}, and that the p47^{phox} C terminus and the p67^{phox} region from the first to the second SH3 domains play negative regulatory roles in the oxidase activation.

Inter- and Intra-molecular Interactions of the N-terminal SH3 Domain of p47^{phox}—To investigate the molecular event that enables p47^{phox} to activate the oxidase, we compared the nature of the C terminally deleted p47^{phox} (p47-ΔC) with that of the full-length one (p47-F). It is well established that, upon cell stimulation, p47^{phox} interacts with the C-terminal cytoplasmic tail of p22^{phox} (5, 8–11, 13–15). The induced interaction is mediated by the N-terminal SH3 domain of p47^{phox} and is essential for the oxidase activation (11, 15). An *in vitro* binding assay using purified proteins revealed that p47-ΔC directly bound to p22^{phox} as strongly as the p47^{phox} composed solely of the SH3 domains (p47-(SH3)₂) did, whereas the full-length p47^{phox} could not (Fig. 5A). The result completely agrees with that obtained by an *in vivo* binding assay in the yeast two-

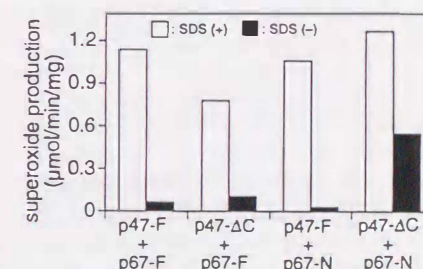


FIG. 4. Requirement of both active p47^{phox} and p67^{phox} for the anionic amphiphile-independent oxidase activation. p47-F (6.5 μg/ml) or p47-ΔC (6.7 μg/ml) were incubated with p67-F (5.9 μg/ml) or p67-N (7.2 μg/ml) in the presence or absence of 100 μM SDS, in the reaction mixture containing the neutrophil membrane, Rac2, GTPγS, and FAD. The superoxide producing activity was determined as described under "Experimental Procedures."

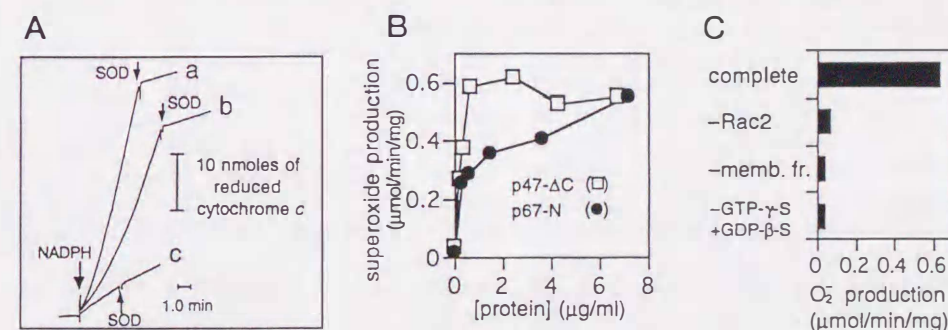


FIG. 3. Anionic amphiphile-independent activation of the NADPH oxidase *in vitro*. A, spectrophotometric records of superoxide production monitored by ferricytochrome c reduction. The mixture containing human neutrophil membrane (10.2 μg/ml), Rac2 (35.8 μg/ml), GTPγS (10 μM), and FAD (10 μM) were incubated with p47-F (6.5 μg/ml) and p67-F (5.9 μg/ml) (traces a and c) or with p47-ΔC (6.7 μg/ml) and p67-N (7.2 μg/ml) (trace b) in the presence (trace a) or absence (traces b and c) of 100 μM SDS. The reaction was initiated by the addition of 1 mM NADPH, and terminated by the addition of 10 μg/ml superoxide dismutase (SOD). B, dose dependence of the SDS-independent superoxide production on p47-ΔC and p67-N. C, the NADPH oxidase activity was determined under various conditions; the complete system, without the membrane, without Rac2, and without GTPγS but with 1.0 mM GDPβS.

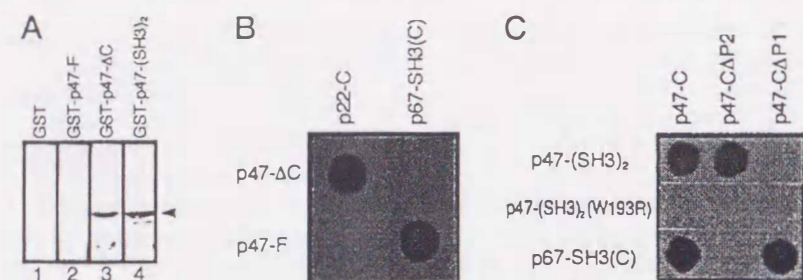


FIG. 5. Intermolecular and intramolecular interactions of p47^{phox}. **A**, *in vitro* binding of p47-ΔC, but not p47-F, with p22^{phox}. GST-p22-C was subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with the indicated GST-fused p47^{phox}. Lane 1, GST; lane 2, GST-p47-F; lane 3, GST-p47-ΔC; lane 4, GST-p47-(SH3)₂. The arrow indicates GST-p22-C. **B**, *in vivo* interaction of p47-ΔC, but not p47-F, with p22^{phox}. The yeast strain SFY256 was cotransformed with pairs of recombinant plasmids pGAD424 encoding p47-ΔC or p47-F, and pGBT9 encoding p22-C or p67-SH3(C). All possible pairs between the pGAD and pGBT plasmids were tested for activation of *lacZ* reporter genes examined by the β-galactosidase filter assay. **C**, *in vivo* interaction of the p47^{phox} N-terminal SH3 domain with p47-C. The yeast strain SFY256 was cotransformed with pairs of pGAD-p47-(SH3)₂, p47-(SH3)₂(W193R), or -p47-F, and pGBT9-p47-C, -p47-ΔC2, or -p47-ΔC1. All possible pairs between the pGAD and pGBT plasmids were tested for activation of *lacZ* reporter genes examined by the β-galactosidase filter assay.

hybrid system (Fig. 5B). In the C terminally deleted protein, thus, the N-terminal SH3 domain exists in a state accessible to the target p22^{phox}. This raised a question how the SH3 domain is normally masked. We have previously shown that the SH3 domains of p47^{phox} can interact with the C terminus of this protein, an interaction which seems to keep the SH3 domains inaccessible (5). To determine the precise regions involved in this interaction, we performed binding experiments in the two-hybrid system. The N-terminal SH3 domain seems responsible for the interaction with the C terminus (p47-C; amino acid residues 286–390), since a mutation in this domain (Trp¹⁹³-Arg) abrogated the binding (Fig. 5C) but one in the other SH3 domain (Trp²⁶³-Arg) did not (data not shown). The results are consistent with the observation that the N-terminal SH3 domain can interact with p47^{phox} *in vitro* (15). As shown in Fig. 5C, the SH3 domain interacted with p47-ΔC2 that lacked the PRR (Pro³⁶¹-Gln-Pro-Ala-Val-Pro-Pro-Arg-Pro³⁶⁹), the target for the C-terminal SH3 domain of p67^{phox} (6, 8, 12). Another deletion (the deleted residues 299–346), giving p47-ΔC1, abolished the interaction with the p47^{phox} SH3 domain, but did not affect the contact with p67^{phox} (Fig. 5C). Thus the intramolecular target of the p47^{phox} N-terminal SH3 domain seems different from the site for the p67^{phox} SH3 domain. The p67^{phox} binding site is likely to be exposed in the folded inactive form of p47^{phox}, as indicated by the two hybrid interaction between the full-length p47^{phox} and the C-terminal SH3 domain of p67^{phox} (Fig. 5B).

DISCUSSION

Here we presented that the phagocyte NADPH oxidase is activated by the C terminally deleted p47^{phox} (p47-ΔC) and the N terminus of p67^{phox} (p67-N), even in the absence of *in vitro* activators, anionic amphiphiles, such as arachidonic acid and SDS. The observation that the anionic amphiphile-independent activation requires both active forms of p47^{phox} and p67^{phox} indicates that the two proteins are the primary targets of the activators in the cell-free system. On the other hand, the action of the amphiphiles on either cytochrome *b*₅₅₈ or Rac, if any, is not essential for the oxidase activation. The extent of the oxidase activation using both truncated proteins is comparable to that elicited by the amphiphiles: about a half of the superoxide producing activity is obtained in the present system. To our knowledge, a similar level of the activation has not been accomplished in any reported cell-free systems using purified cytosolic factors without the amphiphiles. Two recent studies have demonstrated arachidonic acid- or SDS-independent cell-free activation of the NADPH oxidase using crude neutrophil cytosol (28, 29): one reports that phosphatidic acid fully activates the enzyme in a phosphorylation-dependent manner in a

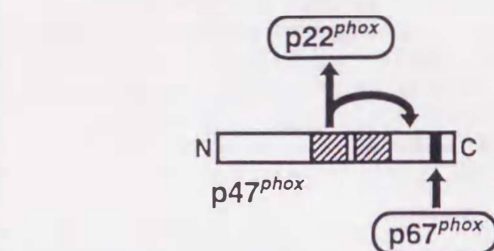


FIG. 6. Schematic representation of intermolecular and intramolecular interactions of p47^{phox}. The hatched and solid boxes represent the SH3 domains and the PRR of p47^{phox}, respectively. The arrows indicate the SH3 domain-mediate interactions. For details, see text.

system using cellular membranes and cytosol with diacylglycerol (28), and the other shows that phosphorylated p47^{phox} is capable of activating the oxidase, but to a lesser extent, when membranes are treated with cytosol and GTPγS (29).

The present system is considered quite useful for studying in detail the activation mechanisms of the individual cytosolic proteins. In the absence of the amphiphiles, the oxidase activation is dependent on the state of p47^{phox}, when p67-N (an active form of p67^{phox}) is used with the GTP-bound Rac2. Under the conditions, the oxidase is activated by the addition of the active p47^{phox} (p47-ΔC), but not the full-length one (Fig. 4). Similarly, with p47-ΔC, the state of p67^{phox} is the determinant. When both p47-ΔC and p67-N are present, the activation totally depends on the state of Rac: little superoxide production was observed with Rac2 in the GDP-bound inactive form (Fig. 3C).

We also studied here the mechanism for activating p47^{phox}. The active p47^{phox} (p47-ΔC) interacted with p22^{phox} both *in vivo* and *in vitro*, while the full-length one in the resting state did not (Fig. 5). This interaction is mediated via binding of the p47^{phox} N-terminal SH3 domain to the PRR of p22^{phox} (11, 15), an interaction which is indispensable for the oxidase activation (11, 15). Thus the conformational change of p47^{phox} that is induced by the amphiphiles appears to culminate in unmasking of the N-terminal SH3 domain, leading to the access to p22^{phox}. In the resting state, this domain is masked probably by interacting with a C-terminal region of p47^{phox}. The region required for this interaction is different from the PRR, the target for the C-terminal SH3 domain of p67^{phox}. This implies that the intramolecular interaction in p47^{phox} and its intermolecular binding to p67^{phox} are not mutually exclusive, *i.e.* both interactions can occur at the same time. Indeed the full-length p47^{phox}, in which the N-terminal SH3 domain is masked, can bind to the p67^{phox} SH3 domain (Fig. 5B). The intramolecular interaction appears to require the target-binding surface of the

p47^{phox} SH3 domain, since a mutation of the surface (W193R) abrogates the binding (Fig. 5C). On the other hand, the target region in p47^{phox} does not contain a typical PRR. In this context, it should be noted that the SH3 domain of the tyrosine kinase Src interacts intramolecularly, via its target-binding surface, with the region lacking a proline-rich sequence (30). Taken together, the N-terminal SH3 domain of p47^{phox} likely undergoes an intramolecular interaction with the C-terminal region, which could restrict access of the domain to its target (Fig. 6).

The anionic amphiphile activators seem to disrupt the intramolecular interaction in p47^{phox}, thereby liberating the SH3 domain to engage p22^{phox}. When cells are stimulated, arachidonic acid released from the membrane may promote the conformational change of p47^{phox}, as well as p67^{phox}, leading to the superoxide production. It is known that, during activation, p47^{phox} becomes phosphorylated at multiple sites in the C-terminal region (31), accompanied by its translocation to the membrane, and it has recently been reported that the phosphorylated p47^{phox} is active in a cell-free activation system (29). Future studies should test whether the phosphorylation converts the intramolecular interaction of the p47^{phox} SH3 domain to the intermolecular one with p22^{phox}, thereby activating the oxidase. In addition, the present finding that the truncated versions of p47^{phox} and p67^{phox} are both active, also raises a possibility that these proteins might be proteolytically activated in stimulated cells. Some protease inhibitors are shown to inhibit the superoxide production by phagocytes (32).

This study unequivocally shows that the amphiphile activators directly interact not only with p47^{phox} but also with p67^{phox}, the latter interaction which is previously suggested (33, 34). The precise event evoked in p67^{phox}, however, is presently unknown. The oxidase activation is repressed by the p67^{phox} region containing both SH3 domains. Pinpointing the responsible region will help our understanding of the mechanism, and such studies are now in progress in our laboratory.

On the basis of the studies on the NADPH oxidase system, we have previously proposed (5) and advanced here the "masking-unmasking" model for a regulatory mechanism of SH3 domain-mediated interactions: an SH3 domain, that is normally masked via its intramolecular interaction, becomes exposed to intermolecularly interact with the target. It should be strengthened that, in the oxidase system, the induced intermolecular interaction is specific and required for the activation both *in vivo* and *in vitro* (5, 8, 9, 11, 15): p22^{phox} is the bona fide target for the N-terminal SH3 domain of p47^{phox}. A similar molecular event has recently been postulated in the T-cell specific tyrosine kinase Itk, a member of the Tec family of non-receptor tyrosine kinase, that is required for signaling via the T-cell antigen receptor (35). The PRR adjacent to the SH3 domain of Itk interacts with the domain intramolecularly. Formation of this complex prevents the SH3 domain and the PRR from interacting with their respective putative substrates, Sam68 and Grb2 (35). In p120 Ras-GAP (GTPase-activating protein), containing an SH3 domain flanked by two SH2 domains, it undergoes a conformational change that leads to increased accessibility of the target binding surface of its SH3 domain, when two closely linked phosphotyrosine-containing peptides bind simultaneously to the two SH2 domains (36). Furthermore, recent structural studies have revealed that the SH3 domain of the tyrosine kinases Src and Hck interacts intramolecularly not only with the linker region between the SH2 and kinase domains, but also simultaneously with the kinase domain, resulting in inhibition of the enzymatic activity (30, 37). Thus such regulatory intramolecular association of SH3 domains, as occurred in p47^{phox}, is currently considered to

be much more general than previously expected (38). This type of the regulation would be found in a variety of signaling proteins that carry both an SH3 domain and a PRR, such as the p85 subunit of phosphoinositide 3-kinase (39) and the yeast protein Bem1p (40).

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