# Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase

(superoxide/cytochrome $b_{558}$/proline-rich region/chronic granulomatous disease)

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ABSTRACT    The phagocyte NADPH oxidase, dormant in resting cells, is activated during phagocytosis to produce superoxide, a precursor of microbicidal oxidants. The activated oxidase is a complex of membrane-integrated cytochrome $b_{558}$, composed of 91-kDa (gp91$^{phox}$) and 22-kDa (p22$^{phox}$) subunits, and two cytosolic factors (p47$^{phox}$ and p67$^{phox}$), each containing two Src homology 3 (SH3) domains. Here we show that the region of the tandem SH3 domains of p47$^{phox}$ (p47-SH3) expressed as a glutathione S-transferase fusion protein inhibits the superoxide production in a cell-free system, indicating involvement of the domains in the activation. Furthermore, we find that arachidonic acid and sodium dodecyl sulfate, activators of the oxidase in vitro, cause exposure of p47-SH3, which has probably been masked by the C-terminal region of this protein in a resting state. The unmasking of p47-SH3 appears to play a crucial role in the assembly of the oxidase components, because p47-SH3 binds to both p22$^{phox}$ and p67$^{phox}$ but fails to interact with a mutant p22$^{phox}$ carrying a Pro-156 → Gln substitution in a proline-rich region, which has been found in a patient with chronic granulomatous disease. Based on the observations, we propose a signal-transducing mechanism whereby normally inaccessible SH3 domains become exposed upon activation to interact with their target proteins.

During ingestion of microbes or upon stimulation with various soluble molecules, neutrophils and other phagocytic cells produce superoxide ($O_2^-$), a precursor of microbicidal oxidants (1–4). The process involves activation of the phagocyte NADPH oxidase, dormant in resting cells, that catalyzes reduction of molecular oxygen to superoxide in conjunction with oxidation of NADPH. The significance of the NADPH oxidase in host defense is made evident by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD) whose phagocytes lack the superoxide-producing system (1–4).

The active NADPH oxidase is found on the phagocyte membrane as an enzyme complex, the components of which are identified as targets of genetic defects causing CGD. The one identified at an earlier stage is a phagocyte-specific membrane-integrated b-type cytochrome, cytochrome $b_{558}$ (5–11), composed of 91-kDa and 22-kDa subunits (designated gp91$^{phox}$ and p22$^{phox}$, respectively). The cytochrome is now considered to be a flavocytochrome comprising an apparatus transporting electrons from NADPH via FAD and then heme to molecular oxygen (12–16). Identification of other oxidase components has advanced by development of a cell-free activation system reconstituted with both membrane and cytosolic fractions of phagocytes in the presence of arachidonic acid or sodium dodecyl sulfate (SDS) (17). Three cytosolic proteins are required for the cell-free activation of the oxidase, two of which (designated p47$^{phox}$ and p67$^{phox}$) have been identified by their absence in patients with CGD (18–21). In addition to these specialized factors, as a third cytosolic factor, the small GTP-binding protein p21$^{rac}$ (rac 1 and/or rac 2) is also involved in the system (22–24).

Although the components of the NADPH oxidase are thus identified, little is known about the mechanism for their assembly leading to activation of the enzyme. Upon phagocyte stimulation, the cytosolic components translocate to the membrane where cytochrome $b_{558}$ resides (25, 26). Experiments using neutrophils from CGD patients have revealed that the translocation of p47$^{phox}$ and p67$^{phox}$ requires interaction between p47$^{phox}$ and cytochrome $b_{558}$ (25). Neither p47$^{phox}$ nor p67$^{phox}$ translocates to the membrane in stimulated neutrophils that lack cytochrome $b_{558}$, indicating that the cytochrome provides a membrane docking site for the cytosolic components. In patients with p47$^{phox}$ deficiency, p67$^{phox}$ fails to migrate, whereas p47$^{phox}$ does bind to the membrane in stimulated neutrophils from patients deficient in p67$^{phox}$. Thus p47$^{phox}$ seems to play a central role in the assembly of these components on the membrane.

The molecular mechanism by which p47$^{phox}$ mediates the assembly of the oxidase components remains to be investigated. The protein p47$^{phox}$, as well as p67$^{phox}$, contains two Src homology 3 (SH3) domains (21). SH3 domains are present in many signaling proteins and have recently been shown to mediate interactions with proteins containing proline-rich regions (27–32). We focused on the role of the SH3 domains of p47$^{phox}$ in assembly and activation of the phagocyte NADPH oxidase.

In the present study, we show that activation of the phagocyte NADPH oxidase involves interaction through the SH3 domains of p47$^{phox}$. Arachidonic acid and SDS, activators of the oxidase in vitro, cause exposure of the SH3 domains of p47$^{phox}$, which seem to have been masked by the C-terminal region of the protein in a dormant state. Furthermore, we find that the exposed SH3 domains of p47$^{phox}$ bind to both p22$^{phox}$ and p67$^{phox}$ and that a point mutation in p22$^{phox}$ (Pro-156 → Gln substitution in a proline-rich region), which has been reported in a patient with CGD (33), results in loss of the interaction. Based on the observations, we propose a signal-transducing mechanism through SH3 domains: normally inaccessible SH3 domains become exposed upon activation to interact with their target proteins.
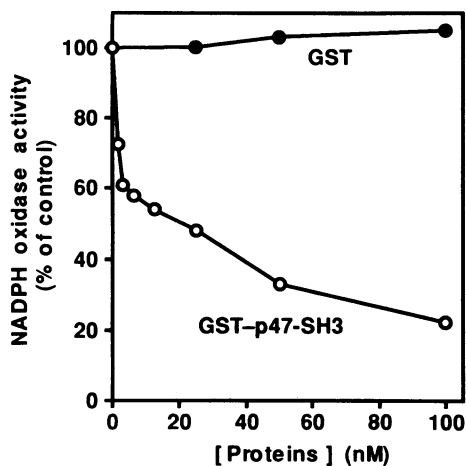
Abbreviations: CGD, chronic granulomatous disease; SH3, Src homology 3; GST, glutathione S-transferase.
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FIG. 1.   Inhibition of NADPH oxidase activity by GST–p47-SH3 in the cell-free system. Human neutrophil NADPH oxidase was activated in the presence of GST–p47-SH3 or GST alone. The rate of superoxide production in the absence of the GST proteins was 383 ± 7 nmol/min per mg of membrane protein ($n = 6$), which was set as 100%. Percentage of rates relative to the control is plotted against the concentrations of GST–p47-SH3 (○) or GST alone (●). The values represent the averages of two independent experiments.

## MATERIALS AND METHODS

**Glutathione S-Transferase (GST) Fusion Proteins.** DNA fragments encoding p47-SH3 (amino acid residues 154–285 of p47phox), p47-N (amino acid residues 1–153), and p47-C (amino acid residues 286–390) were amplified by polymerase chain reaction (PCR) from a cloned cDNA encoding human p47phox, using specific 3' and 5' primers that included restric-

tion sites for BamHI and EcoRI. The PCR products were subcloned into the pGEX-2T expression vector (Pharmacia). The cDNA encoding the cytoplasmic domain of human p22phox (amino acid residues 132–195), designated as p22 (132–195), was obtained by PCR from a human neutrophil cDNA library. For the fragment encoding p22 (132–195, P156Q), the mutation was introduced into p22 (132–195) by PCR mutagenesis. The DNA fragment encoding p22 (145–170) was constructed by PCR of p22 (132–195). The PCR fragments were cloned in-frame into the BamHI and EcoRI sites of the pGEX-2T vector. The DNA fragments encoding p22 (151–160) and p22 (151–160, P156Q), were derived from synthetic oligonucleotides, which were cloned into the BamHI and EcoRI sites of the pGEX-2T vector. Sequencing of the constructs to confirm their identity was done with Sequenase according to the manufacturer's protocol (United States Biochemical). The fusion protein was expressed in *Escherichia coli* and purified by glutathione-Sepharose beads (Pharmacia) according to the manufacturer's protocol.

**Assay of NADPH-Dependent Superoxide Production.** Membrane and cytosolic fractions of human neutrophils were prepared as described (34). The assay mixture was composed of 100 mM potassium phosphate (pH 7.0), 75 μM cytochrome $c$, 10 μM FAD, 10 μM GTP[γS], 1.0 mM EGTA, 1.0 mM MgCl₂, and 1.0 mM NaN₃. The cytosol (220 μg/ml) and membrane (15 μg/ml) were mixed with the indicated concentration of GST–p47-SH3 or GST alone, followed by incubation with an optimal concentration of arachidonic acid (50 μM) or of SDS (100 μM) for 2 min at room temperature. The reaction was initiated by addition of NADPH (250 μM) to the reaction mixture, and NADPH-dependent superoxide-producing activity was measured by determining the rate of superoxide dismutase-inhibitable ferricytochrome $c$ reduc-
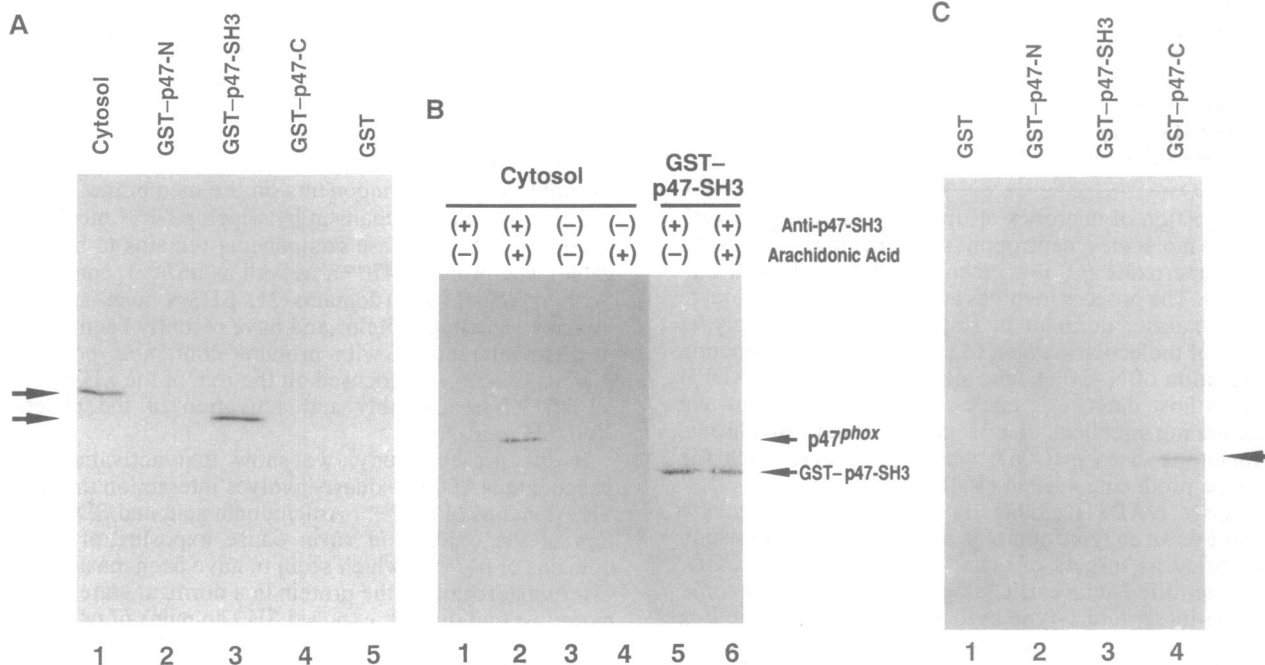


FIG. 2.   Unmasking of the SH3 domains of p47phox. (*A*) The monoclonal antibody 5-13 recognizes the SH3 domains of p47phox. Proteins (lane 1, human neutrophil cytosol; lane 2, GST–p47-N; lane 3, GST–p47-SH3; lane 4, GST–p47-C; lane 5, GST alone) were subjected to SDS/PAGE and immunoblotted using the monoclonal antibody 5-13. The upper and lower arrows indicate the positions of p47phox and GST–p47-SH3, respectively. (*B*) SDS-dependent binding of the monoclonal antibody 5-13 to p47-SH3. Human neutrophil cytosol (lanes 1–4) or GST–p47-SH3 (lanes 5 and 6) was incubated with (lanes 3–6) or without (lanes 1 and 2) the monoclonal antibody in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 50 μM arachidonic acid. Immune complexes were separated on SDS/PAGE and probed with the biotinylated monoclonal antibody 5-13 followed by streptoavidin-conjugated horseradish peroxidase. (*C*) Binding of p47-SH3 to the C-terminal region of p47phox. GST fusion proteins (lane 1, GST alone; lane 2, GST–p47-N; lane 3, GST–p47-SH3; lane 4, GST–p47-C) were subjected to SDS/PAGE and probed with biotinylated GST–p47-SH3 and subsequently with streptoavidin-conjugated horseradish peroxidase. The arrow shows the position of GST–p47-C.

tion at 550–540 nm with a dual-wavelength spectrophotometer (Hitachi 557).

**Immunoblot Analyses.** The monoclonal antibody 5-13 was raised against recombinant human p47phox that was produced in Sf9 insect cells (35). Human neutrophil cytosol (110 μg) or purified GST-fusion proteins (2.5 μg) were applied to 10% SDS/PAGE gel and then transferred to nitrocellulose membrane. The membrane was blocked in buffer A (500 mM NaCl/20 mM Tris, pH 7.5) containing 3% non-fat dry milk for 2 hr at room temperature. The filter was probed with the monoclonal antibody 5-13 in buffer A plus 0.25% gelatin for 2 hr at room temperature and washed three times with buffer A plus 0.1% Tween 20. Complexes were detected using anti-mouse IgG antibodies.

**Binding of the Monoclonal Antibody 5-13 to p47phox.** Human neutrophil cytosol (200 μg) or GST–p47-SH3 (2 μg) was incubated in buffer B (100 mM potassium phosphate, pH 7.0/10 μM GTP[γS]/1.0 mM EGTA/1.0 mM MgCl₂) with or without the monoclonal antibody 5-13 in the presence or absence of 50 μM arachidonic acid for 5 min at room temperature. Immune complexes were precipitated by incubation with protein G-Sepharose beads for 1 hr at 4°C and the beads were washed four times by phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄. Proteins eluted from the beads with Laemmli sample buffer were subjected to SDS/PAGE on 10% gel and immunoblotted using the biotinylated monoclonal antibody 5-13 and streptoavidin-conjugated horseradish peroxidase.

**Binding of p47-SH3 to p47-C and to the Cytoplasmic Domain of p22phox.** The GST fusion proteins (10 pmol), purified by glutathione-Sepharose beads, were subjected to 10% SDS/PAGE and transferred to nitrocellulose membrane. The membrane was blocked by buffer A containing 3% non-fat dry milk for 2 hr at room temperature. Biotinylated GST–p47-SH3 (3 μg/ml) was added to the same buffer, incubated for 1 hr at room temperature, and washed four times with buffer A containing 0.1% Tween 20. The membrane was incubated in buffer A plus 0.25% gelatin with streptoavidin-conjugated horseradish peroxidase for 2 hr at room temperature and developed.

**Binding of p47-SH3 to p67phox.** Human neutrophil cytosol (800 μg) was incubated in buffer B with 200 pmol of GST alone or GST–p47-SH3 in the presence or absence of 50 μM arachidonic acid for 5 min at room temperature. Glutathione-Sepharose beads were added to each mixture and incubated for 1 hr at 4°C to retrieve the GST fusion proteins, which were then washed four times by PBS. The proteins eluted from the beads with Laemmli sample buffer were subjected to SDS/PAGE on a 10% gel and immunoblotted using a monoclonal antibody raised against recombinant human p67phox that was produced in Sf9 insect cells (35).

## RESULTS AND DISCUSSION

To investigate roles of the SH3 domains of p47phox, we produced and isolated the fusion protein GST–p47-SH3 and tested its effect on the NADPH oxidase in the presence of 50 μM arachidonic acid (Fig. 1) or 100 μM SDS (data not shown) in a cell-free system. It is well established that these anionic amphiphiles act as activators *in vitro* (17), probably by interacting with components of the oxidase. GST–p47-SH3 inhibited superoxide production in a dose-dependent manner, while GST alone did not affect the production at all (Fig. 1). This suggests that a protein–protein interaction mediated via p47-SH3 is involved in the activation process of the phagocyte NADPH oxidase.

The involvement of p47-SH3 in the oxidase activation implies that p47phox may be a target of arachidonic acid and SDS. Interactions with these substances may cause a conformational change in p47phox, allowing the SH3 domains to associate with other oxidase factors. To test this possibility,
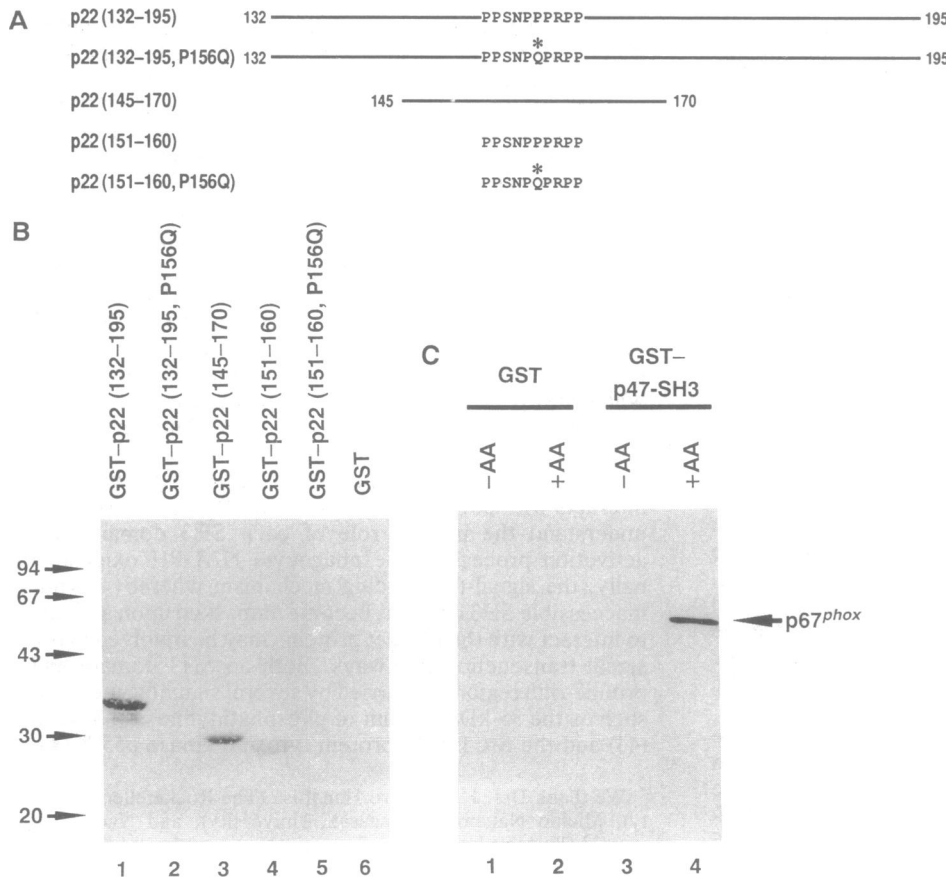


FIG. 3. Binding of the GST–p47-SH3 fusion protein to the cytoplasmic domain of p22phox and to p67phox. (*A*) Schematic representation of the cytoplasmic domain of p22phox and smaller fragments used to study the p47phox binding site in p22phox. (*B*) Binding of p47-SH3 to the cytoplasmic domain of p22phox. The GST fusion proteins were subjected to SDS/PAGE, transferred to nitrocellulose membrane, and probed with the biotinylated GST–p47-SH3. Lane 1, GST–p22 (132–195); lane 2, GST–p22 (145–195); lane 3, GST–p22 (145–170); lane 4, GST–p22 (151–160); lane 5, GST–p22 (151–160, P156Q); lane 6, GST alone. Molecular size markers are indicated in kDa. (*C*) Arachidonic acid-dependent binding of GST–p47-SH3 to p67phox. Human neutrophil cytosol was incubated with GST alone (lanes 1 and 2) or GST–p47-SH3 (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 50 μM arachidonic acid (AA). Complexes were precipitated with glutathione-Sepharose beads and immunoblotted using a monoclonal antibody against p67phox.
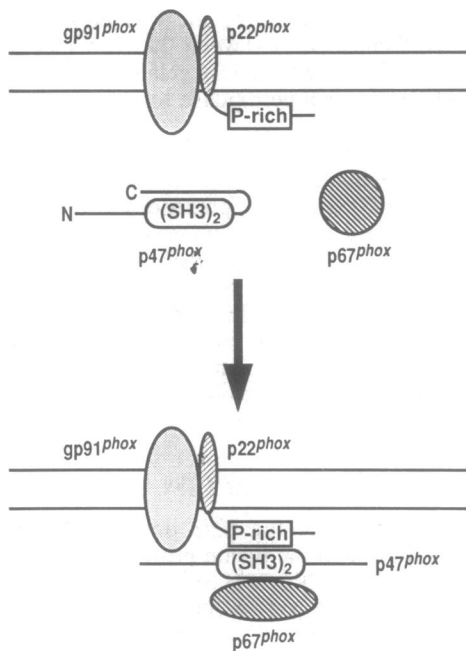
FIG. 4. Model for the activation mechanism of the phagocyte NADPH oxidase. Stimulation of the cells causes exposure of the SH3 domains of p47$^{phox}$ that have been masked by the C-terminal region of this protein. Then the unmasked SH3 domains interact with the proline-rich regions of both p22$^{phox}$ and p67$^{phox}$. A similar conformational change may also occur in p67$^{phox}$. The interaction leads to formation of the active NADPH oxidase complex.

we used a monoclonal antibody that specifically recognizes p47-SH3 (Fig. 2*A*). Binding of the anti-p47-SH3 antibody to native p47$^{phox}$ is totally dependent on the presence of arachidonic acid (Fig. 2*B*) or SDS (data not shown). When GST–p47-SH3 was used instead of native p47$^{phox}$, the recognition of the SH3 domains by the antibody was independent of arachidonic acid (Fig. 2*B*) or SDS (data not shown), indicating that these activators did not alter the conformation of the antibody or of p47-SH3 under the condition used. These results strongly suggest that p47-SH3 is normally inaccessible to the antibody but becomes exposed upon addition of the activators. Several recent studies have shown that SH3 domains interact with proline-rich regions of their target proteins (28–32). In fact, p47$^{phox}$ itself contains a proline-rich stretch in its C-terminal region. As shown in Fig. 2*C*, this region clearly possessed the ability to bind to p47-SH3. The result suggests that, in the absence of the activators, p47-SH3 is probably masked by the C-terminal region of p47$^{phox}$ through an intramolecular cis interaction.

It is likely that the unmasked p47-SH3, in turn, interacts with other oxidase factors to form the active enzyme complex. The large subunit of cytochrome $b_{558}$ (gp91$^{phox}$) does not appear to contain a binding site for p47-SH3, since it lacks amino acid stretches rich in proline (5–7). Also, the cytosolic small GTP-binding protein p21$^{rac}$, which is implicated in the oxidase activation in addition to p47$^{phox}$ and p67$^{phox}$ (22–24), and the regulators of p21$^{rac}$ (*rho* GDI and *smg* GDS) (36, 37) are unlikely to bind to p47-SH3 for the same reason. On the other hand, proline-rich regions do exist in the cytoplasmic domain of p22$^{phox}$ (11, 38) and the other specialized cytosolic factor p67$^{phox}$ (21).

We first tested the ability of p47-SH3 to bind to the cytoplasmic domain of p22$^{phox}$ expressed as a GST fusion protein (Fig. 3 *A* and *B*). GST–p47-SH3 strongly bound to the cytoplasmic domain, while the binding was completely abolished by a single amino acid substitution of Gln for Pro-156 in p22$^{phox}$. This substitution in p22$^{phox}$ has been reported in

a CGD patient whose cytochrome $b_{558}$ is normal in appearance and abundance as determined by visible spectroscopy and by immunoblot analyses but is devoid of activity in the cell-free oxidase activation system (33). Thus, the association of p47$^{phox}$ with p22$^{phox}$ seems to be essential for the activation of the NADPH oxidase. As far as we are aware, there has been no previous report of a genetic disease caused by an impaired protein–protein interaction via an SH3 domain.

To further investigate the importance of the mutated region, we used GST fusion proteins with smaller fragments derived from p22$^{phox}$ (Fig. 3 *A* and *B*). A strong binding was observed with the fusion protein that contained the region of amino acids 145–170 of p22$^{phox}$, the sequence of which is well conserved between human and mouse (38). This region contains a 10-amino acid stretch very rich in proline residues (PPSNPPPRPP, in single-letter amino acid code) resembling the peptide (APTMPPPLPP) that mediates binding of 3BP1 to the SH3 domain of c-*Abl* (30). p47-SH3 bound to this 10-amino acid core of p22$^{phox}$, but to a lesser extent. Again, the substitution of Gln for Pro-156 (PPSNPQPRPP) resulted in loss of the binding activity. The proline-rich segment thus directly interacts with p47-SH3. The lower binding affinity of the segment may be due to either its altered structure relative to the larger peptides or the absence of another region that contributes to SH3 binding cooperatively with the proline-rich segment.

We next studied the ability of p47-SH3 to interact with the other cytosolic factor p67$^{phox}$, which also carries proline-rich stretches. Although p67$^{phox}$ failed to interact with p47-SH3 in the absence of arachidonic acid, the addition of arachidonic acid (Fig. 3*C*) or SDS (data not shown) caused binding of p67$^{phox}$ to p47-SH3. The activator-dependent binding of p67$^{phox}$ suggests that the conformation of p67$^{phox}$ may also be changed upon activation by a mechanism similar to that for p47$^{phox}$, because p67$^{phox}$ contains SH3 domains as well.

Fig. 4 summarizes the proposed activation mechanism of the phagocyte NADPH oxidase. The activation involves a conformational change of p47$^{phox}$. Arachidonic acid and SDS break a cis interaction of p47-SH3 with the C-terminal region of this protein, leading to trans interactions with both p22$^{phox}$ and p67$^{phox}$. In the intact cells, the unmasking of p47$^{phox}$ may be caused by a protein modification, such as phosphorylation. Stimulation of the cells indeed causes phosphorylation of p47$^{phox}$, which appears to be correlated with its translocation to membrane (39, 40). Alternatively, arachidonic acid, released from membrane phospholipid upon stimulation, might act as a second messenger to activate p47$^{phox}$ directly (41). In this case, it is possible that arachidonic acid also interacts with other proteins. A recent study shows that biologically active lipids including arachidonic acid are capable of disrupting complexation of *rho* GDI with p21$^{rac}$, which may play a role in modulating the activity of p21$^{rac}$ and thus the NADPH oxidase activity (42). Another unsolved question in the present study is which one of the two SH3 domains of p47$^{phox}$ interacts with p22$^{phox}$ and which one interacts with p67$^{phox}$. Further investigations are required to understand the precise role of each SH3 domain in the activation process of the phagocyte NADPH oxidase. Finally, the signal-transducing mechanism whereby normally inaccessible SH3 domains become unmasked upon activation to interact with their target proteins may be involved in other signal transduction pathways. Both an SH3 domain and a proline-rich region are carried by several signaling molecules, such as the 85-kDa subunit of phosphatidylinositol 3'-kinase (43) and the Src family protein tyrosine kinase p55$^{blk}$ (44).

1. Cross, A. R. & Jones, O. T. G. (1991) *Biochim. Biophys. Acta* **1057**, 281–298.
2. Smith, R. M. & Curnutte, J. T. (1991) *Blood* **77**, 673–686.
3. Babior, B. M. (1992) *Adv. Enzymol.* **65**, 59–95.
4. Segal, A. W. & Abo, A. (1993) *Trends Biochem. Sci.* **18**, 43–47.
5. Royer-Pokora, B., Kunkel, L. M., Monaco, A. P., Goff, S. C., Newburger, P. E., Boehner, R. L., Cole, F. S., Curnutte, J. T. & Orkin, S. H. (1986) *Nature (London)* **322**, 32–38.
6. Dinauer, M. C., Orkin, S. H., Brown, R., Jesaitis, A. J. & Parkos, C. A. (1987) *Nature (London)* **327**, 717–720.
7. Teahan, C., Rowe, P., Parker, P., Totty, N. & Segal, A. W. (1987) *Nature (London)* **327**, 720–721.
8. Nakamura, M., Murakami, M., Koga, T., Tanaka, Y. & Minakami, S. (1987) *Blood* **69**, 1404–1408.
9. Rotrosen, D., Yeung, C. L. & Katkin, J. P. (1993) *J. Biol. Chem.* **268**, 14256–14260.
10. Knoller, S., Shpungin, S. & Pick, E. (1991) *J. Biol. Chem.* **266**, 2795–2804.
11. Parkos, C. A., Dinauer, M. C., Walker, L. E., Allen, R. A., Jesaitis, A. J. & Orkin, S. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3319–3323.
12. Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L. & Kwong, C. H. (1992) *Science* **256**, 1459–1462.
13. Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H. & Scrace, G. (1992) *Biochem. J.* **284**, 781–788.
14. Sumimoto, H., Sakamoto, N., Nozaki, M., Sakaki, Y., Takeshige, K. & Minakami, S. (1992) *Biochem. Biophys. Res. Commun.* **186**, 1368–1375.
15. Koshkin, V. & Pick, E. (1993) *FEBS Lett.* **327**, 57–62.
16. Doussiere, J., Brandolin, G., Derrien, V. & Vignais, P. V. (1993) *Biochemistry* **32**, 8880–8887.
17. Bromberg, Y. & Pick, E. (1985) *J. Biol. Chem.* **260**, 13539–13545.
18. Nunoi, H., Rotrosen, D., Gallin, J. I. & Malech, H. L. (1988) *Science* **242**, 1298–1301.
19. Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I. & Malech, H. L. (1989) *Science* **245**, 409–412.
20. Volpp, B. D., Nauseef, W. M., Donelson, J. E., Moser, D. R. & Clark, R. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7195–7199.
21. Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M. G., Nauseef, W. M., Clark, R. A., Gallin, J. I. & Malech, H. L. (1990) *Science* **248**, 727–730.
22. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G. & Segal, A. W. (1991) *Nature (London)* **353**, 668–670.
23. Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T. & Bokoch, G. M. (1991) *Science* **254**, 1512–1515.
24. Mizuno, T., Kaibuchi, K., Ando, A., Musha, T., Hiraoka, K., Takaishi, K., Asada, M., Nunoi, H., Matsuda, I. & Takai, Y. (1992) *J. Biol. Chem.* **267**, 10215–10218.
25. Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H. & Clark, R. A. (1991) *J. Clin. Invest.* **87**, 352–356.
26. Park, J.-W., Ma, M., Ruedi, J. M., Smith, R. M. & Babior, B. M. (1992) *J. Biol. Chem.* **267**, 17327–17332.
27. Mayer, B. J. & Baltimore, D. (1992) *Trends Cell Biol.* **3**, 8–13.
28. Bridge, R. B. & Hanafusa, H. (1993) *Science* **262**, 1522–1524.
29. Cicchetti, P., Mayer, B. J., Thiel, G. & Baltimore, D. (1992) *Science* **257**, 803–806.
30. Ren, R., Mayer, B. J., Cicchetti, P. & Baltimore, D. (1993) *Science* **259**, 1157–1161.
31. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. & Bowtell, D. (1993) *Nature (London)* **363**, 83–85.
32. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D. & Waterfield, M. D. (1993) *Cell* **75**, 25–36.
33. Dinauer, M. C., Pierce, E. A., Erickson, R. W., Muhlebach, T. J., Messner, H., Orkin, S. H., Seger, R. A. & Curnutte, J. T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11231–11235.
34. Nozaki, M., Takeshige, K., Sumimoto, H. & Minakami, S. (1990) *Eur. J. Biochem.* **187**, 335–340.
35. Leto, T. L., Garrett, M. C., Fujii, H. & Nunoi, H. (1991) *J. Biol. Chem.* **266**, 19812–19818.
36. Fukumoto, Y., Kaibuchi, K., Hori, Y., Fujioka, H., Araki, S., Ueda, T., Kikuchi, A. & Takai, Y. (1990) *Oncogene* **5**, 1321–1328.
37. Kaibuchi, K., Mizuno, T., Fujioka, H., Yamamoto, T., Kishi, K., Fukumoto, Y., Hori, Y. & Takai, Y. (1991) *Mol. Cell. Biol.* **11**, 2873–2880.
38. Sumimoto, H., Nozaki, M., Sasaki, H., Takeshige, K., Sakaki, Y. & Minakami, S. (1989) *Biochem. Biophys. Res. Commun.* **165**, 902–906.
39. Heyworth, P. G., Shrimpton, C. F. & Segal, A. W. (1989) *Biochem. J.* **260**, 243–248.
40. Rotrosen, D. & Leto, T. L. (1990) *J. Biol. Chem.* **265**, 19910–19915.
41. Henderson, L. M., Chappell, J. B. & Jones, O. T. G. (1989) *Biochem. J.* **264**, 249–255.
42. Chuang, T.-H., Bohl, B. P. & Bokoch, G. M. (1993) *J. Biol. Chem.* **268**, 26206–26211.
43. Lu, X., Marengere, L. M., Koch, C. A. & Pawson, T. (1993) *Mol. Cell. Biol.* **13**, 5225–5232.
44. Pleiman, C. M., Clark, M. R., Timson, G., Winitz, S., Coggeshall, K. M., Johnson, G. L., Shaw, A. S. & Cambier, J. C. (1993) *Mol. Cell. Biol.* **13**, 5877–5887.