An SH3 domain-mediated interaction between the phagocyte NADPH oxidase factors p40^{phox} and p47^{phox}

Takashi Ito[a,*], Rika Nakamura[a,b], Hideki Sumimoto[c,**], Koichiro Takeshige[c], Yoshiyuki Sakaki[a]

[a]*Human Genome Center, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan*
[b]*Department of Biochemical Genetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113, Japan*
[c]*Department of Biochemistry, Kyushu University School of Medicine, Higashi-ku, Fukuoka 812, Japan*

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Abstract The phagocyte NADPH oxidase is activated during phagocytosis to produce superoxide, following assembly of a membrane-integrated cytochrome b_{558} with cytosolic proteins, p47^{phox}, p67^{phox} and p40^{phox}, each containing Src homology 3 (SH3) domains. While both p47^{phox} and p67^{phox} are indispensable for the oxidase activity, role of p40^{phox} remains obscure. Here we study interaction between p40^{phox} and p47^{phox} by two independent methods, a two-hybrid system in the yeast and an in vitro binding assay using purified proteins. The present results show that the interaction is mediated via binding of the SH3 domain of p40^{phox} to a C-terminal proline-rich region of p47^{phox}. This proline-rich region is also the target for binding of p67^{phox}, and the SH3 domain of p40^{phox} can inhibit the binding of the C-terminal one of p67^{phox} to p47^{phox}.

Key words: NADPH oxidase; SH3 domain; Proline-rich region

1. Introduction

The phagocyte NADPH oxidase is dormant in resting cells, and activated during phagocytosis to catalyze the one-electron reduction of molecular oxygen in conjunction with oxidation of NADPH to generate superoxide (O_2^-), a precursor of microbicidal oxidants [1,2]. The importance of the oxidase to host immunity is evident from that recurrent severe infection occurs in patients with chronic granulomatous disease (CGD) whose phagocytes are deficient in superoxide production [3].

Activation of the NADPH oxidase involves assembly of cytochrome b_{558}, a membrane-integrated flavocytochrome comprising gp91^{phox} and p22^{phox}, with cytosolic proteins such as p47^{phox}, p67^{phox} and the small G-protein Rac (Rac1 and/or Rac2) [1,2]. The factors that reside in the cytosol of resting cells, upon activation translocate to the membrane to form a complex with cytochrome b_{558} [1,2]. The translocation of p47^{phox} and p67^{phox} requires interaction between p47^{phox} and cytochrome b_{558}; p47^{phox} carries p67^{phox} into an active oxidase complex where the cytochrome provides a docking site [4,5].

Both p47^{phox} and p67^{phox} contain two Src homology 3 (SH3) domains. The domain is present in many signaling proteins and known to mediate interactions via binding to proline-rich regions in target proteins [6,7]. Current evidence indicates that SH3 domains function in assembly of the NADPH oxidase factors [8-11]. An SH3 domain of p47^{phox} appears to be critical for interaction with cytochrome b_{558}; it binds to the C-terminal cytoplasmic domain of p22^{phox} but fails to interact with a mutant p22^{phox} carrying Pro^{156}Gln substitution in a proline-rich region, which is found in a patient with CGD [8,10]. On the other hand, the C-terminal SH3 domain of p67^{phox} is involved in binding to p47^{phox} [9-11].

The protein p40^{phox} containing an SH3 domain was originally identified as one that tightly associates with p67^{phox} in resting phagocytes [12-14], but its role is poorly understood. Although p40^{phox} as well as p47^{phox} and p67^{phox} seems to translocate to the membrane upon cell stimulation depending on the presence of cytochrome b_{558} [13], the NADPH oxidase activity can be reconstituted without p40^{phox} under cell-free activation conditions [15,16], suggesting that p40^{phox} may play a role in modulation of the activity. A recent study using the yeast two-hybrid system has shown that the C-terminal portion of p40^{phox} but not its SH3 domain is involved in association with p67^{phox}, and that a region containing the SH3 domain of p40^{phox} can interact with p47^{phox} [17]. Little is, however, known about interaction between p40^{phox} and p47^{phox}, including the question which site of p47^{phox} is responsible for the binding to p40^{phox}.

As a step for understanding the role of p40^{phox}, we focused on interaction between p40^{phox} and p47^{phox}, since p47^{phox} seems to play a central role in assembly of the NADPH oxidase factors. The present results obtained by two independent methods, the two-hybrid system and an in vitro binding assay using expressed proteins, indicate that the interaction is mediated via binding of the SH3 domain of p40^{phox} to a C-terminal proline-rich region of p47^{phox}, a region which is also the target for the p67^{phox} binding.

2. Materials and methods

2.1. Two-hybrid experiments

The vectors and strains used for two-hybrid experiments were obtained from Clontech Lab., Inc. (CA, USA). The multiple cloning sites of pGAD424 and pGBT9 were modified so that the inserts from glutathione *S*-transferase (GST) fusion plasmids (pGEX-2T, Pharmacia Biotech) can be readily transferred in correct orientation and reading frames. Human cDNAs encoding the N-terminal (amino acid residues 1–167), SH3 (amino acid residues 168–233) and C-terminal domains (amino acid residues 234–339) of p40^{phox} were amplified from differentiated HL60 RNAs by RT-PCR and cloned into the *Bam*HI and *Eco*RI sites of the modified GAL4 activation domain vector, pGAD424g. The obtained plasmids were named pGAD::p40-N, pGAD::p40-SH3 and pGAD::p40-C, respectively. A mutant p40-SH3 carrying Trp^{207}Arg substitution was made by

**Corresponding author. Fax: (81) (3) 5449-5445.

**Corresponding author. Fax: (81) (92) 632-2373.

Abbreviations: CGD, chronic granulomatous disease; SH3, Src homology 3; GST, glutathione *S*-transferase; MBP, maltose binding protein

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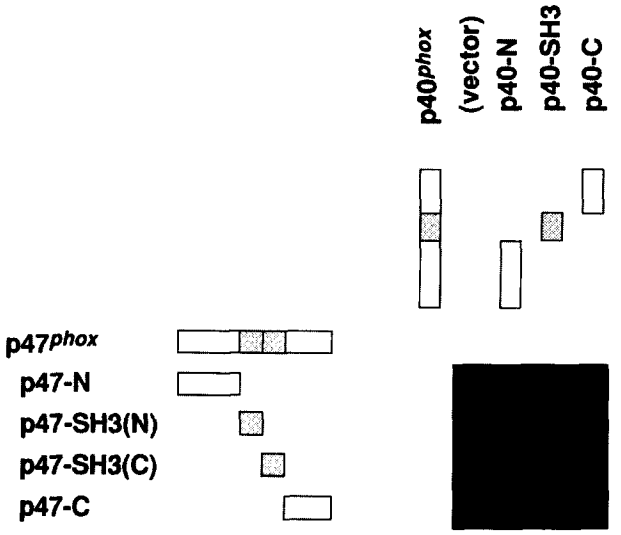

Fig. 1. Interaction of $p40^{phox}$ with $p47^{phox}$ analyzed by the yeast two-hybrid system. The yeast strain HF7c was cotransformed with pairs of recombinant plasmids pGAD424 and pGBT9, the former encoding the indicated domain of $p40^{phox}$ fused to the GAL4 activation domain and the latter encoding the indicated domain of $p47^{phox}$ fused to the GAL4 DNA-binding domain. All the possible pGAD424 and pGBT9 plasmid pairs were tested for the His^+ phenotype. The structure of domains and intact proteins is schematically illustrated. Shaded boxes indicates SH3 domains. (vector) indicates the cloning vector used as a negative control.

PCR-directed mutagenesis and ligated to pGAD424g to yield pGAD::p40-SH3(W207R). Human cDNAs encoding N-terminal (amino acid residues 1–153), SH3(N) (amino acid residues 154–219), SH3(C) (amino acid residues 223–286), and C-terminal domains (amino acid residues 286–390) of $p47^{phox}$ were transferred from corresponding GST-fusion constructs [8] to the modified GAL4 DNA-binding domain vector, pGBT9g, thereby constructing pGBT::p47-N, pGBT::p47-SH3(N), pGBT::p47-SH3(C) and pGBT::p47-C, respectively. Deletion mutants of pGAD::p47-C, namely p47-CΔP1, p47-CΔP2 and p47-CΔP1P2, were lacking amino acid residues 299–346, 360–390, and 341–368, respectively. The domains of $p47^{phox}$, N-terminal (amino acid residues 1–153), $(SH3)_2$ (amino acid residues 154–286) and C-terminal domains (amino acid residues 286–390), were also transferred from corresponding GST-fusion constructs [8] to pGAD424g, constructing pGAD::p47-N, pGAD::p47-$(SH3)_2$ and pGAD::p47-C, respectively. Similarly, from the corresponding inserts of pGEX-2T (H.S., unpublished), four plasmids for human $p67^{phox}$ were constructed, namely pGBT::p67-N (amino acid residues 1–232), pGBT::p67-SH3(N) (amino acid residues 228–291), pGBT::p67-IS (amino acid residues 291–450) and pGBT::p67-SH3(C) (amino acid residues 445–516). The C-terminal SH3 domain of $p67^{phox}$ was also ligated to pGAD424g, constructing pGAD::p67-SH3(C). These plasmids were used following the confirmative DNA sequencing.

All the possible pairs between the pGAD and pGBT plasmids were cotransformed into competent yeast HF7c cells with *HIS3* and *lacZ* reporter genes using a modified lithium-acetate method [18]. Following the selection for Leu^+ and Trp^+ phenotype, transformants were tested for their ability to grow on plates lacking histidine. These indicator plates were supplemented with 10 mM 3-aminotriazole to suppress the background growth due to leaky expression of HIS3 gene in HF7c cells. Activation of *lacZ* reporter was examined by the filter assay according to the manufacturer's recommendation.

For the competitive two-hybrid assay, cDNA encoding the C-terminal SH3 domain of $p67^{phox}$ was cloned into a newly constructed activation domain vector pGAF (T.I., unpublished), a derivative of pGAD424g carrying *URA3*, instead of *LEU2*, as the selection marker, to obtain pGAF::p67-SH3(C). To express the SH3 domain of $p40^{phox}$ as the competitor, its cDNA fragment was cloned into the *MluI* site, but not the conventional cloning sites, of pACT2, a high-expression type activation domain vector (Clontech Lab., Inc., CA, USA), to obtain pACTΔ::p40-SH3. This construct directs the expression of the SH3 domain fused to an intact nuclear localization signal and a part of the GAL4 activation domain, which shows no transcription activation. The plasmids pGBT::p47-CΔP1 and pGAF::p67-SH3(C) were cotransformed with pACT2 or pACTΔ::p40-SH3 into Y187z strain (T.I., unpublished), a *ura3* derivative of Y187. Following the selection of triple transformants for Trp^+, Ura^+ and Leu^+ phenotype, the activation of *lacZ* reporter was examined by the filter method.

2.2. In vitro binding assay using purified proteins

The maltose binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLab, MA, USA) was also modified in its multiple cloning site to facilitate the transfer of inserts from pGEX-2T plasmids. The modified vector, pMAL-c2g, was used for the expression of the three regions of $p40^{phox}$. Expressed proteins were purified using amylose resin (New England BioLab, MA, USA). The individual domains of $p47^{phox}$ were expressed as GST fusion proteins and purified using glutathione Sepharose beads (Pharmacia Biotech) as described previously [8]. The purified GST fusion proteins were subjected to 9% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Following the blocking with non-fat dry milk, the filters were incubated with MBP fusion proteins and washed as described previously [8]. Then, the washed filters were probed with anti-MBP antibody (New England BioLab, MA, USA), and the complex was detected using secondary anti-mouse IgG antibodies conjugated with peroxidase (Sigma) and a POD immunostain kit (Wako, Osaka, Japan) according to the manufacturer's instructions.

3. Results and discussion

In the present study on interaction between $p40^{phox}$ and $p47^{phox}$, we first used a two-hybrid system in the yeast. Fuchs et al., using the system, have recently shown that the region containing the SH3 domain of $p40^{phox}$ and its adjacent regions (amino acids 146–260) is involved in interaction with $p47^{phox}$, leaving the target on the latter unclear [17]. To clarify whether the interaction requires the SH3 domain itself or its adjacent regions, we prepared a construct comprising solely the SH3

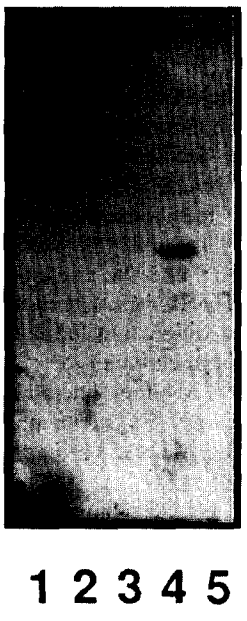

Fig. 2. In vitro interaction between $p40^{phox}$ and $p47^{phox}$. The four domains of $p47^{phox}$ expressed as GST fusion proteins and GST were subjected to SDS-PAGE, and transferred to a nitrocellulose filter: GST-p47-N (lane 1), GST-p47-SH3(N) (lane 2), GST-p47-SH3(C) (lane 3), GST-p47-C (lane 4) and GST (lane 5). The filter was subsequently overlaid with the SH3 domain of $p40^{phox}$ fused to MBP as a probe.

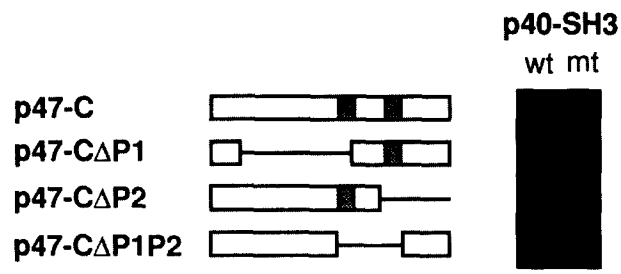

Fig. 3. Mapping of the p40^{phox} SH3 domain binding site on p47^{phox}. The vector pGAD424 encoding wild-type SH3 domain of p40^{phox} (wt) or its mutant carrying the Trp^{207}Arg substitution (mt) was co-transformed into yeast strain HF7c with the vector pGBT9 encoding the C-terminal portion of p47^{phox} (p47-C) or its deletion mutants (p47-CΔP1, p47-CΔP2, or p47-CΔP1P2). All the possible pGAD424 and pGBT9 plasmid pairs were tested for the His$^+$ phenotype. The structure of p47-C and its derived proteins is schematically illustrated. Proline-rich regions are indicated by shaded boxes.

domain of p40^{phox} (amino acids 168–233) as well as those of the N-terminal and C-terminal domains, and fused them to the activation domain of GAL4 on the vector pGAD424g to obtain pGAD::p40-SH3, pGAD::p40-N and pGAD::p40-C, respectively. On the other hand, for mapping the responsible region of p47^{phox}, the protein was divided into N-terminal, the N-terminal SH3, the C-terminal SH3 and C-terminal domains, each of which was fused to the DNA-binding domain of GAL4 on the pGBT9g vector to construct pGBT::p47-N, pGBT::p47-SH3(N), pGBT::p47-SH3(C) and pGBT::p47-C, respectively. All the possible pairs between the pGAD::p40 and pGBT::p47 plasmids were introduced into competent HF7c cells and tested for activation of *HIS3* reporter gene. As shown in Fig. 1, the strain bearing both pGAD::p40-SH3 and pGBT::p47-C, but not other combinations, grew on the plate lacking histidine. The activation of *lacZ* reporter was confirmed by the filter assay (data not shown). These results indicate that the SH3 domain of p40^{phox} mediates interaction with p47^{phox} via binding to the C-terminal portion.

To confirm that the SH3 domain of p40^{phox} directly interacts with the C-terminal portion of p47^{phox}, we expressed and purified all the domains of both proteins, and used them for an in vitro binding assay: the four domains of p47^{phox} fused to GST were transferred to membrane and probed with the three domains of p40^{phox} tagged with MBP. When the SH3 domain of p40^{phox} was used as a probe, a positive interaction was detected only with the C-terminal portion of p47^{phox} (Fig. 2). On the other hand, the other two domains of p40^{phox} did not interact with any portions of p47^{phox} (data not shown). These results were in complete agreement with the two-hybrid data described above, and we thus conclude that direct binding of the SH3 domain of p40^{phox} to the C-terminal portion of p47^{phox} mediates interaction between these two proteins, but other domains or portions are not involved in the interaction.

To further investigate the nature of the interaction, we introduced a mutation into the SH3 domain of p40^{phox}, causing replacement of Trp-207 by Arg. This tryptophan residue is the most conserved one in SH3 domains [19], and it directly interacts with a proline of target peptides [20,21]. The equivalent mutations (substitution of Arg or Leu for Trp) in Src and Crk result in loss of function [22,23]. The mutant p40^{phox} SH3 domain carrying the Trp^{207}Arg substitution failed to interact with the C-terminal portion of p47^{phox} (Fig. 3), indicating that the interaction occurs in a manner common to those via SH3 domains. As is well established, targets of SH3 domains are proline-rich regions and the minimal requirement is a Pro-Xa-Xa-Pro motif [20]. In the C-terminal portion of p47^{phox}, the motif exists in stretches of Pro^{341}-Gly-Pro-Gln-Ser-Pro-Gly-Ser-Pro^{349} and Pro^{361}-Gln-Pro-Ala-Val-Pro-Pro-Arg-Pro^{369}. To determine which is involved in interaction with the SH3 domain of p40^{phox}, we made three constructs: a p47-C lacking the former stretch (p47-CΔP1); without the latter (p47-CΔP2); deleting both (p47-CΔP1P2). Two-hybrid assays were performed using these constructs (Fig. 3). All of the p47^{phox} fusion proteins that interacted with the SH3 domain of p40^{phox} shared a common proline-rich stretch of Pro^{361}-Gln-Pro-Ala-Val-Pro-Pro-Arg-Pro^{369}, indicating that the SH3 domain of p40^{phox} binds the C-terminal proline-rich region of p47^{phox}.

The C-terminal portion of p47^{phox} seems to be not only the target of p40^{phox} but also that of p67^{phox}. Binding assays in vitro have shown that the C-terminal SH3 domain of p67^{phox} interacts with this portion of p47^{phox} [9,10]. To verify that the interaction occurs in vivo, we carried out two-hybrid experiments. The DNA-binding domain of GAL4 on the pGBT9g

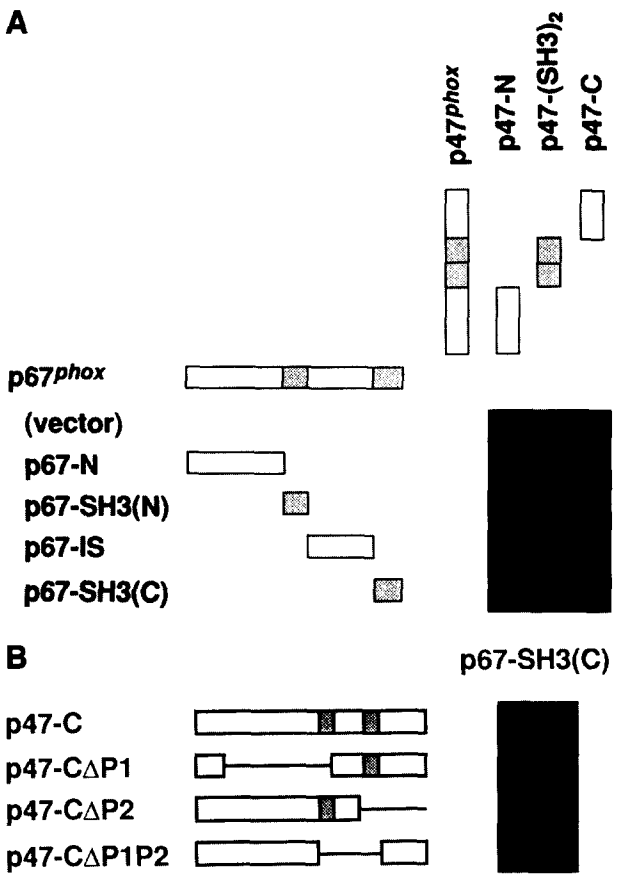

Fig. 4. Interaction of p67^{phox} with p47^{phox}. (A) The four domains of p67^{phox} expressed as fusion with the GAL4 DNA-binding domain were tested for interaction with the three portions of p47^{phox} fused to the GAL4 activation domain, in yeast HF7c cells. (B) Various deletion mutants of the p47^{phox} C-terminal portion (p47-C), as fusion with GAL4 DNA-binding domain, were tested for interaction with the C-terminal SH3 domain of p67^{phox} (p67-SH3(C)) fused to the GAL4 activation domain, in yeast HF7c cells. Interactions between proteins are demonstrated by histidine-independent growth. The structure of domains and intact proteins is schematically illustrated. Shaded boxes indicate SH3 domains (A) or proline-rich regions (B). (vector) indicates the cloning vector used as a negative control.

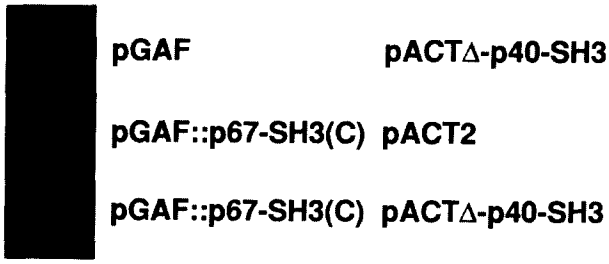

Fig. 5. In vivo inhibition of p67^{phox} binding to p47^{phox} by the SH3 domain of p40^{phox}. The plasmids pGBT::p47-CΔP1 and pGAF::p67-SH3(C) (or pGAF) were cotransfomed with pACT2 or pACTΔ::p40-SH3 into Y187z strain, a *ura3* derivative of Y187. Following the selection of triple transformants for Trp$^+$, Ura$^+$ and Leu$^+$ phenotype, the activation of *lacZ* reporter was examined by the filter method.

vector was fused to the N-terminal portion, the N-terminal SH3 domain, the portion between both SH3 domains (inter-SH3 portion) and the C-terminal SH3 domain of p67^{phox} to obtain pGBT::p67-N, pGBT::p67-SH3(N), pGBT::p67-IS and pGBT::p67-SH3(C), respectively. All the possible pairs between the pGBT::p67 and pGAD::p47 plasmids were tested for activation of the HIS3 reporter gene. Only the strain bearing both pGBT::p67-SH3(C) and pGAD::p47-C showed the His$^+$ phenotype (Fig. 4A). Thus the C-terminal SH3 domain of p67^{phox} interacts with the C-terminal portion of p47^{phox} in vivo as well as in vitro. We next pinpointed the target region on p47^{phox}. As shown in Fig. 4B, strains that grew without histidine all expressed both the C-terminal SH3 domain of p67^{phox} and, as a part of the fusion protein, a proline-rich stretch of Pro^{361}-Gln-Pro-Ala-Val-Pro-Pro-Arg-Pro^{369} on p47^{phox}. This indicates that the target of p67^{phox} is the C-terminal proline-rich region of p47^{phox}, which is consistent with that determined by in vitro binding assays [9,10]. It should be noted that this region was also responsible for binding of p40^{phox} (Fig. 3).

The finding that the same region on p47^{phox} is recognized by the p40^{phox} SH3 domain and by the C-terminal one of p67^{phox} suggests that the two SH3 domains compete with each other in binding to p47^{phox}. To test this possibility, we designed an in vivo competition assay, in which the effect of the overexpressed SH3 domain of p40^{phox} on the interaction between p47^{phox} and p67^{phox} was examined using the yeast two-hybrid system. The interaction between the two was readily detected by *lacZ* reporter activation in the presence of control plasmid pACT2, whereas it was severely impaired when the SH3 domain of p40^{phox} was overexpressed from pACTΔ::p40-SH3 (Fig. 5). Thus the SH3 domain of p40^{phox} can inhibit, probably by competition, the binding of the C-terminal one of p67^{phox} to the proline-rich region of p47^{phox}.

Interaction between p47^{phox} and p67^{phox} in stimulated phagocytes is considered to play a crucial role in forming an active NADPH oxidase complex, since p47^{phox} is indispensable for translocation of p67^{phox} to the membrane where cytochrome b_{558} resides [4]. Our in vivo data (Fig. 4), as well as those obtained in vitro [10], suggest that the interaction is solely mediated via binding of the C-terminal SH3 domain of p67^{phox} to the C-terminal proline-rich region of p47^{phox}. The importance of the C-terminal SH3 domain is also supported by the finding that p67^{phox} deleting this domain fails to restore oxidase activity in Epstein-Barr virus-transformed B lymphocytes that lack p67^{phox} [24]. As shown in this study, the p40^{phox} SH3 domain recognizes and binds the same region on p47^{phox} as the C-terminal SH3 domain of p67^{phox} does. Competition between p40^{phox} and p67^{phox} in binding to p47^{phox} may modulate the NADPH oxidase activity.

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