p40\textsuperscript{phox} Down-regulates NADPH Oxidase Activity through Interactions with Its SH3 Domain*

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The NADPH oxidase of phagocytes generates microbicidal oxidants in response to a variety of stimuli. Its activation and assembly involve multiple SH3 domain interactions among several oxidase components. Here we present evidence that the cytosolic oxidase-associated protein, p40\textsuperscript{phox}, mediates down-regulation of NADPH oxidase through interactions with its SH3 domain. Recombinant p40\textsuperscript{phox} was produced in several eukaryotic expression systems (insect, mammalian, and yeast) to explore its role in oxidase function in relation to domains involved in interactions with other factors, p47\textsuperscript{phox} and p67\textsuperscript{phox}. p40\textsuperscript{phox} inhibited oxidase activity \textit{in vitro} when added to neutrophil membranes and recombinant p47\textsuperscript{phox}, p67\textsuperscript{phox}, and p21rar. Co-transfection of p40\textsuperscript{phox} into K562 cells resulted in significant decreases (−40%) in whole cell oxidase activity. Furthermore, the isolated SH3 domain of p40\textsuperscript{phox} was even more effective in inhibiting whole cell oxidase activity, consistent with experiments showing that this domain binds to the same proline-rich target in p47\textsuperscript{phox} (residues 358–390) that interacts with p67\textsuperscript{phox}. In contrast, deletion of the carboxy-terminal domain of p40\textsuperscript{phox} that binds to p67\textsuperscript{phox} did not relieve its oxidase inhibitory effects. Thus, p40\textsuperscript{phox} appears to down-regulate oxidase function by competing with an SH3 domain interaction between other essential oxidase components.

Production of superoxide anion by phagocytic blood cells involves assembly of an activated NADPH oxidase complex of membrane-bound and cytosolic components (for review, see Ref. 1). Five distinct NADPH oxidase components have been identified; deficiencies or defects in any one of four essential proteins result in impaired oxidase activity associated with chronic granulomatous disease (CGD)\textsuperscript{1} (1). Two are subunits of cytochrome b\textsubscript{558} (p22\textsuperscript{phox} and gp91\textsuperscript{phox}) that donate electrons to molecular oxygen to yield superoxide, and two are cytosolic proteins, p47\textsuperscript{phox} and p67\textsuperscript{phox}, that associate with the membrane-bound flavocytochrome during oxidase activation. The fifth essential component is a Ras-related GTPase, p21rar, that renders the enzyme sensitive to guanine nucleotides (2–4). Recently, yet another factor, p40\textsuperscript{phox}, was identified in a high molecular weight cytosolic complex with p47\textsuperscript{phox} and p67\textsuperscript{phox} (5–7). The importance of the p40\textsuperscript{phox}-p67\textsuperscript{phox} association is evident in resting neutrophils from p67\textsuperscript{phox}-deficient CGD patients, where the absence of p67\textsuperscript{phox} was correlated with reduced levels of p40\textsuperscript{phox} (5, 7–9).

The deduced structure of p40\textsuperscript{phox} reveals homology with p67\textsuperscript{phox} and p47\textsuperscript{phox} (5), which both contain two Src homology 3 (SH3) domains that also occur in various intracellular signaling proteins in eukaryotes (10, 11). The SH3 domain of p40\textsuperscript{phox} has its greatest homology to the second SH3 domain of p67\textsuperscript{phox} (44% identity), although p40\textsuperscript{phox} and p47\textsuperscript{phox} also exhibit homology in regions N-terminal to their SH3 domains. Recently, the role of SH3 domains in NADPH oxidase function has been the subject of intense investigation (12–23). Several SH3 interactions have been shown to participate in translocation and assembly of the active membrane-bound enzyme in whole cells (12, 13, 16–18). The first p47\textsuperscript{phox} SH3 domain engages a membrane-bound proline-rich target in p22\textsuperscript{phox} (17), while the second SH3 domain of p67\textsuperscript{phox} binds to a carboxy-terminal p47\textsuperscript{phox} target and is critical for p67\textsuperscript{phox} membrane translocation (12–14, 16–19). Recent work has shown that the SH3 domain of p40\textsuperscript{phox} also binds the same carboxy-terminal target in p47\textsuperscript{phox} that binds p67\textsuperscript{phox} (21, 22). Other SH3 domain interactions have been demonstrated, including intramolecular contacts within p47\textsuperscript{phox} (12, 15–17); several of these associations may be regulated during oxidase activation.

Despite work in several laboratories on the interactions between oxidase components, the function of p40\textsuperscript{phox} has remained unclear (5–9, 21–25). Antibodies against p40\textsuperscript{phox} were shown to inhibit NADPH oxidase \textit{in vitro} (25), although the effects of p40\textsuperscript{phox} on oxidase activity have not been examined directly, and CGD patients with lesions in the p40\textsuperscript{phox} gene have not been described. Furthermore, p40\textsuperscript{phox} is not needed to reconstitute high levels of oxidase activity in cell-free systems containing purified p21rar, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and relipidated flavocytochrome b\textsubscript{558} (26, 27). Like several of the core oxidase components, p40\textsuperscript{phox} expression is myeloid-specific (5). The fact that p40\textsuperscript{phox} is bound in a complex with p67\textsuperscript{phox} and p47\textsuperscript{phox} that translocates to the membrane upon activation suggests that p40\textsuperscript{phox} could have a role in modulating the respiratory burst. Here we report about interactions of p40\textsuperscript{phox} with other oxidase components and explore the effects of these interactions on oxidase activity, both in cell-free and whole cell reconstituted systems. Based on these observations, we provide evidence for yet another SH3 domain-mediated interaction, which in this case appears to inhibit oxidase function.

**EXPERIMENTAL PROCEDURES**

**Recombinant Protein Expression**—The full coding sequence of p40\textsuperscript{phox} was cloned from a human leukocyte cDNA library (Clontech, Palo Alto, CA; catalog No. HL4014AB) using the polymerase chain reaction. Amplification primers were targeted to sequences beginning 21 bases upstream from the start codon and ending 50 bases after the stop codon of the p40\textsuperscript{phox} cDNA (5). The p40\textsuperscript{phox} polymerase chain reaction product was first cloned into the TA cloning vector (Invitrogen, San Diego, CA) for sequencing and then subcloned (EcoRI fragment) into pGEX3X for production of recombinant p40\textsuperscript{phox}-glutathione S-transferase (GST) fusion protein in \textit{Escherichia coli}. The fusion protein was affinity purified.
from induced bacteria on glutathione-Sepharose as described elsewhere (28).

Production of recombinant p40phox baculovirus for infection of cultured Sf9 (Spodoptera frugiperda) cells was performed by methods described earlier (29), with the following modifications. The p40 cDNA was subcloned into a baculovirus transfer vector (pAC-rac2) by digesting the HindIII-EcoRI segment of p40phox cDNA with EcoRI and BamHI and inserting it into the EcoRI site of pAC-rac2. The recombinant baculovirus was plaque-purified and multiplied by infecting large scale suspension cultures (100 ml) grown at 2.5 °C.

Antiserum Preparation—The p40phox fusion protein preparations from inclusion bodies were subjected to SDS-PAGE and visualized by staining with Coomassie blue. The full-length protein was excised and used to immunize rabbits (primary immunization, 150 μg in complete Freund’s adjuvant, followed by two boosts in incomplete Freund’s adjuvant at 2-week intervals). Subsequent boosts used affinity-purified baculovirus-derived p40phox eluted from SDS-PAGE gels. The antibody obtained after 10 weeks of immunization cross-reacted specifically with p40phox in neutrophil cytosol and recombinant p40phox from Sf9 cells.

Mammalian Expression Vectors—The p40phox cDNA was excised from the TA-p40 vector with KpnI (5’) and XbaI (3’) and subcloned into the episomal vector pCEP4 (Invitrogen) restricted with KpnI and NcoI. The carboxyl-terminal-deleted form of p40phox (residues 1–260) was constructed by restriction of full-length cDNA with KpnI (5’ polylinker) and BglII (internal) sites and subcloned into KpnI and BamHI-digested pCEP4. The segment encoding the SH3 domain of p40phox (residues 293–465) was polymerase chain reaction amplification. pREP episomal expression vectors carrying p47phox and p67phox cDNAs were described earlier (13, 30).

Functional Studies in Transfected Cells—Co-transfection of pCEP or pREP plasmids containing p67phox, p47phox, and p40phox cDNAs (20 μg each) into transfected K562 (gp91phox-expressing) cells was performed by electroporation as described previously (16, 30). In control experiments, pCEPACAT vector (Invitrogen) was used in place of pCEP40. At 48 h post-transfection, 106 cells/ml were selected for 5 days in complete medium containing 50 μg/ml hygromycin B. Production of p40phox was monitored by hygromycin-resistant cells was confirmed by immunoblotting of cytosolic fractions with rabbit p40 antisera (1:1000 dilution). All functional assays were performed within 2 weeks of hygromycin selection. Whole cell superoxide production in response to PMA stimulation (2 μM) was determined as the superoxide dismutase-inhibitable chemiluminescence signal (31). Transfection efficiency was scaled up to purify p40phox from baculovirus-infected Sf9 cells (72 h post-infection) contained a predominant 40-kDa band in neutrophil cytosol and recombinant p40phox produced in Sf9 cells was subcloned (EcoRI and BglII fragments) into an appropriately cut pGADGH vector. Expression vectors were confirmed by DNA sequence and in some cases by Western blotting. All manipulations in yeast strains HF7c or CG1945 were according to manufacturers’ directions. Interactions between fusion proteins were monitored by histidine prototrophy and β-galactosidase activity detected with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside within host colonies that were adsorbed onto nitrocellulose filters (32). All constructs were co-expressed along with appropriate control (nonrecombinant or empty) vectors to test for false positive signals.

Cell-free NADPH Oxidase (Cytochrome c Reduction) Assay—Cell-free superoxide production was determined from the superoxide dismutase-inhibitable reduction of cytochrome c as described earlier (13, 29). Reactions (100 μl) contained varying amounts of Sf9 cell cytosols derived from uninfected or p40phox baculovirus-infected cultures (2 μg/ml) along with 5 × 105 cell equivalents of deoxycholate-solubilized neutrophil membranes, 0.8 μg each of recombinant p47phox, and p67phox (29) and 1 μg of the mutant form of p21rac (Q61L) preloaded with GTPγS. This mutant form of p21rac was expressed in pGex-2T(4) and was more active than wild-type p21rac as noted earlier (29). Negative control reactions contained 5 μg of superoxide dismutase, in which case cytochrome c reduction did not exceed 5% of the activity observed in the absence of superoxide dismutase. Maximum rates of superoxide production were calculated from a linear least-squares fit of 5 consecutive 1-min data points based on reactions performed in duplicate.

RESULTS

Expression of Recombinant p40phox—Attempts to isolate intact recombinant p40phox from E. coli were not successful due to the proteolytic susceptibility of GST-p40phox fusion protein. The affinity-purified fusion protein contained a predominant 40-kDa band in in vitro cell lysates. Expression of GST-p40phox fusion protein was purified from soluble inclusion bodies of induced cells by preparative SDS-PAGE and used for primary immunization of rabbits against p40phox.

The baculovirus expression system was used for production of native, full-length recombinant p40phox, since earlier work showed that this system was efficient in production of other cytosolic oxidase proteins (29). Sonicated lysates from p40phox baculovirus-infected SF9 cells (72 h post-infection) contained a prominent 40-kDa species (Fig. 1A), which demonstrated immuno-cross-reactivity with a 40-kDa band in neutrophil cytosol (Fig. 1B). The SF9 lysates were considerably enriched in p40phox in comparison with neutrophil cytosol on a specific weight basis. These SF9 preparations were used without further purification as a source of p40phox in subsequent cell-free oxidase reconstitution studies. Baculovirus-derived p40phox also exhibited binding to immobilized GST fused with the proline-rich C-terminal (35-residue) segment of p47phox, while not binding to unfused GST (Fig. 1C), consistent with interactions detected in the yeast two-hybrid system (21, 22) (see below). This affinity system was scaled up to purify p40phox used in later immunizations.

Recombinant p40phox was also expressed in the cell line K562. These cells lack the neutrophil oxidase components p47phox, p67phox, and p40phox and proved to be an efficient transducing model for reconstitution of NADPH oxidase following transduction with gp91phox retrovirus and co-transfection of p47phox and p67phox cDNAs in Epstein-Barr virus-based episomal expression vectors (pREP4 or pREP10) (16, 30). To confirm expression of p40phox in K562 cells, protein lysates from hygromycin-resistant K562 cells transfected with pCEP40phox or control vectors were compared by p40phox immunoblotting. Endogenous p40phox was not detected in K562
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FIG. 1. Characterization of recombinant p40phox produced in baculovirus-infected Sf9 cells. A, Coomassie blue-stained SDS-PAGE (10%) of cytosolic protein fractions (10 μg) from p40phox baculovirus-infected (p40/BV/Sf9) or uninfected (UI/Sf9) insect cells. B, Immunoblotting with anti-p40phox. Protein lysates (5 μg) described in A were transferred to nitrocellulose and probed with anti-p40phox serum detected by enhanced chemiluminescence. PMN, human neutrophil cytosol. C, binding of p40phox to the proline-rich COOH terminus of p47phox (residues 358–390) fused to GST (GST-p47PR). p40phox-containing S9 cytosol (p40/BV/Sf9) was incubated for 20 min with ~5 μg of fused or unfused GST proteins immobilized on glutathione-agarose followed by separation of bound protein complexes (Bd) from total cytosol as described (25). Top, fast green (total protein) staining of nitrocellulose blot after SDS-PAGE. Bottom, immuno-detection of p40phox on the same blot. Arrowheads indicate migration of p40phox; sizes of molecular weight standards are indicated on the right in kilodaltons.

FIG. 2. Production of recombinant p40phox in transfected K562 cells. Cells were co-transfected with various episomal vectors and selected as described under “Experimental Procedures.” Cytoplasmic protein lysates (40 μg) were subjected to SDS-PAGE, electroblotted, and probed with anti-p40phox serum using enhanced chemiluminescence methods. Lanes 1 and 2, lysates from single transfections with pCEP4CAT (control) or pCEP4p40, respectively; lane 3, lysate from K562 cells co-transfected with p40phox, p47phox, and p67phox cDNAs; lane 4, lysate from K562 cells co-transfected with p47phox and p67phox cDNAs; lane 5, human neutrophil cytosol. Sizes of molecular weight standards are indicated on the right in kilodaltons.

FIG. 3. Effects of p40phox on PMA-stimulated superoxide production in co-transfected K562 cells. Superoxide released by 0.5 × 106 K562 cells following stimulation with 2 μg/ml PMA was measured by chemiluminescence as described under “Experimental Procedures” and expressed as relative chemiluminescence units (RLU). gp91phox-transduced K562 cells were co-transfected (20 μg each) with pREP4/p47phox, pREP10/p67phox, and either pCEP4CAT control (L) or pCEP4p40phox (M). Results are representative of data from four transfection experiments. Inset, immunoblot analysis of recombinant p47phox and p67phox produced in pCEP4CAT control (lane 1) and pCEP4p40phox (lane 2) transfected cells.

Whole Cell Oxidase Activity—Retroviral gp91-transduced K562 cells were co-transfected by electroporating 20 μg each of pREP4/p47phox, pREP10/p67phox, and pCEP4/p40phox (or pCEP4CAT (control)) plasmids. Immunoblotting of protein lysates from hygromycin-resistant cells confirmed expression of all three recombinant oxidase proteins in these experiments (Figs. 2 and 3). Oxidase activity in response to PMA activation was monitored by chemiluminescence. Four independent co-transfection experiments were performed and the results of one representative experiment are shown in Fig. 3. A significant inhibition of oxidase activity was observed in the presence of p40phox typically resulting in 35–40% reduction in chemiluminescence signals in comparison to control transfections with pCEP4CAT (Fig. 3). The extent of oxidase inhibition attributed to p40phox expression was significant when considering that this vector lacks a unique selection marker. Since the levels of p47phox and p67phox detected were comparable in cells expressing p40phox or CAT (Fig. 3, inset), variable expression of the other oxidase components did not account for the reduced oxidase activity in the presence of p40phox. While the yield of active oxidase in the presence of p40phox based on the magnitude of the chemiluminescence peak was consistently diminished, the overall kinetic profiles of PMA-elicited respiratory bursts appeared to be similar to control reactions, showing maximum activity 5–10 min after stimulation.

Cell-free NADPH Oxidase Reconstitution Studies—The effects of p40phox on NADPH oxidase activity reconstituted in vitro were examined following preincubation of recombinant baculovirus-derived p40phox lysates with one or the other pure cytosolic factors (p67phox, p47phox). Measurements of superoxide production in a system containing recombinant p47phox, p67phox, and p21rac1 (Q61L) showed a significant dose-dependent inhibition of oxidase activity associated with increasing amounts of p40phox Sf9 cell lysates, in comparison with the same quantities of lysates from uninfected Sf9 cells (Fig. 4). These inhibitory effects were evident irrespective of the order of addition of the other cytosolic factors shown to bind p40phox Predictably, low levels of oxidase inhibition (<25%) were also seen with excessive amounts of control uninfected lysates, although these effects were likely due to effects of high protein concentrations on free arachidonate levels.

Mapping of Interacting Domains within p40phox p47phox and p67phox using the Yeast 2-hybrid System—The yeast two-hybrid system was used to delineate interacting domains within cyto-
solic oxidase proteins that were shown to exist as a complex in
resting neutrophils (5–7). This system has advantages over
other in vitro binding assays, since the interactions between
proteins occurs in vivo and no protein purification or renatur-
ation from SDS-PAGE gels is required (31–33). While the sys-
tem permits qualitative assessment of weak protein-protein
interactions ($K_d < 10^{-8}$ m), quantitative binding data are not
obtained by this approach. The p40phox cDNA sequences
corresponding to various domains tested were subcloned into ac-
tivation domain vectors pGADGH or pGAD424. Coding se-
quences for p67phox and p47phox domains were engineered in
frame with the Gal4 binding domain in pGBT9. As summarized
in Table I, a positive interaction was observed between full-
length p40phox and p67phox, which was abolished by deletion of
80 residues from the carboxyl-terminal end of p40phox
(p403CT). Consistent with this, residues 260–340 (CT) of
p40phox were sufficient to observe an interaction with full-
length p67phox. The corresponding domain of p67phox that
interacts with p40phox was identified within a 172-residue seg-
ment (residues 293–465) bounded by the two SH3 domains,
consistent with two hybrid studies reported recently (22).
The interaction between p40CT and residues 293–465 of
p67phox was also confirmed when these cDNAs were expressed
in the reciprocal vectors (data not shown). There was no evi-
dence for interaction between the isolated SH3 domain of
p40phox and full-length p67phox or p67NT (residues 1–246),
although a weak positive signal detected in a prolonged β-ga-
lactosidase assay was observed with co-expression of full-
length p40phox and the amino-terminal domain (246 residues)
of p67phox (NT) or a smaller fragment (residues 155–246, data
not shown). Others have characterized an interaction between
67 NT and p40phox by surface plasmon resonance, although
binding of residues 293–465 of p67phox with p40phox was not
examined in this case (24).

The interaction between p40phox and p47phox was mapped
to the SH3 domain (residues 172–229) of p40phox and the proline-
rich tail region (residues 351–390) of p47phox consistent with
yeast two-hybrid experiments reported elsewhere (21–23). An
SH3 domain interaction between GRB-2 and human SOS-1
was also demonstrated by yeast two-hybrid expression (33).
The p40phox SH3 domain interaction with the C-terminal do-
main of p47phox was confirmed by expression in reciprocal
vectors (data not shown).

**DISCUSSION**

Previous studies using purified proteins establish five core
NADPH oxidase components as necessary and sufficient for
reconstitution of high levels of oxidase activity in vitro (26, 27).
Recently, p40phox was identified in resting neutrophil cytosol as
yet another protein bound within a high molecular complex
(250 kDa) with two essential cytosolic components, p67
phox, p47phox, and p21rac1 (Q61L), and another protein
bound within a high molecular complex (250 kDa) with two
essential components (p21rac1 (Q61L), p67phox, or p47phox).
Control reactions (C) contained equivalent amounts of uninfected
SF9 cell cytosol in place of p40phox cytosol. Activity is expressed relative to
that observed in the absence of SF9 cytosol (100%), which represented 5.7–
5.9 nmol of superoxide/min in this experiment. The inhibitory effects of
p40phox shown here are consistent with results from four independent
experiments.

**TABLE I**

| Interaction of p40phox with p67phox and p47phox |
|-----------------------------------------------|
| p40phox | Empty vector | p67 FL (res. 1-246) | p67 NT (res. 246-390) | p67 CT (res. 293-465) | p47 CT (res. 351-390) |
| pGBT9 | - | - | - | - | - |
| p40 FL (res. 1-340) | - | + | - | - | - |
| p40 ΔCT (res. 293-465) | - | - | - | - | + |
| p40 SH3 (res. 172-229) | - | - | - | - | + |

**Fig. 4. Effects of recombinant p40phox on cell-free NADPH oxidase activity.** In vitro reconstitution of NADPH oxidase activity was determined from the superoxide dismutase-sensitive reduction of cyto-
tochrome c as described under “Experimental Procedures.” Recombinant
p40phox cytosol was preincubated 20 min at room temperature with
recombinant p47phox ([]), p67phox (○), or p47 NT (□) prior to addition of solubilized
neutrophil membranes, arachidonic acid, substrates, and the other
essential components (p21rac1 (Q61L), p67phox, or p47phox). Control
reactions (C) contained equivalent amounts of uninfected SF9 cell cyto-
sol in place of p40phox cytosol. Activity is expressed relative to that
observed in the absence of SF9 cytosol (100%), which represented 5.7–
5.9 nmol of superoxide/min in this experiment. The inhibitory effects of
p40phox shown here are consistent with results from four independent
experiments.

The yeast two-hybrid system was used to identify interacting do-
mains between p40phox, p67phox, and p47phox. +, positive interaction
between domains tested; −, no interaction observed; +/−, weak β-ga-
lactosidase activity with long incubation.
endogenous protein.

These studies confirm and extend the findings of others by defining precise binding sites between the cytosolic oxidase factors and exploring the role of p40phox in whole cell oxidase function. Work in several laboratories suggested that the primary association in this cytosolic oxidase complex occurs between p67phox and p40phox. In vitro binding studies suggested that the entire pool of cytosolic p40phox was bound in a tight complex with p67phox in resting neutrophils (6, 7). Deficiencies in either p40phox or p67phox seen in autosomal recessive forms of CGD resulted in diminished translocation of p40phox to the membrane (9), although only the patients deficient in p67phox showed dramatically reduced levels of total cellular p40phox, suggesting that p67phox stabilizes p40phox in neutrophils.

Direct interactions have also been demonstrated between p47phox and p40phox, although these interactions were not readily detected by methods involving immobilized proteins, such as surface plasmon resonance or blotting techniques (8, 24). We detected this interaction in solution with recombinant fusion proteins and in the yeast two-hybrid system, as shown by others (21–23). Early work identified p40phox by two-hybrid interactions using p47phox as bait to screen a cDNA library from Epstein-Barr virus-transformed B-cells (23). The importance of the p47phox interaction is evident in our current findings which correlate delineation of binding sites in p40phox with whole cell oxidase function. The p40phox interaction with p47phox was critical for inhibition of NADPH oxidase activity, while deletion of the p67phox binding site in p40phox did not affect its ability to inhibit oxidase activity in whole cells. These observations were not reconciled with recent findings showing in vitro inhibition of the oxidase by antibodies directed against p40phox (C-terminal, p67phox binding domain), although these investigators did not study the proteins directly (25).

The NADPH oxidase inhibition we observed by p40phox is thought to involve the interaction between its SH3 domain and a target within p47phox previously shown to participate in assembly of p67phox in the active oxidase complex (12–14, 16, 18). The isolated SH3 domain of p40phox inhibited whole cell oxidase activity to an extent that exceeded the inhibition by full-length p40phox. The demonstration of an interaction between the SH3 domain of p40phox and the proline-rich carboxyl-terminal domain of p47phox (residues 358–390) was supported by earlier work in which p47phox was shown to bind to a central 115-residue segment of p40phox, half of which included the SH3 domain (22, 23). More recent studies showed the SH3 domain of p40phox binds to the proline-rich tail region of p47phox and thereby inhibits binding of the C-terminal p67phox SH3 domain to the same region of p47phox, when over-expressed in yeast (21). These authors suggested that competitive binding between p40phox and p67phox could modulate oxidase activity. Our observations of inhibitory effects of p40phox or its isolated SH3 domain on whole cell oxidase function lend further support to this competitive binding model.

Our results also provide additional evidence for the importance of the tail-tail interaction between p47phox and p67phox in oxidase activation. The role of this C-terminal domain interaction had been disputed because neither tail domain was necessary for cell-free reconstitution of NADPH oxidase activity (8, 13). However, work in whole transfected cells showed that deletions or mutations in the carboxyl-terminal domains of either protein dramatically affected whole cell oxidase activity and p67phox membrane translocation (13, 16, 18). Since the tail-tail interaction is not essential for cell-free oxidase activity and cell-free oxidase inhibition was not influenced by the order of addition of the three cytosolic proteins, it is unclear whether p40phox inhibits the cell-free system by the same mechanism suggested from the whole cell studies.

In summary, we have examined the role of interactions between p40phox and two other essential oxidase components, both in vitro and in transfected cells where expression of NADPH oxidase components could be genetically manipulated. Based on these findings, we suggest a model outlined in Fig. 6 in which clear distinctions can be made in terms of function between two interactions of p40phox. In one case, the interaction of p40phox with p67phox appears to play no obvious role in the oxidase activation process, although its importance is evident in neutrophils where p40phox stability is affected by the absence
of p67phox seen in CGD (5, 7–9). In the second, the interaction between p47phox and p40phox is not readily detected in resting neutrophil cytosol but was shown to play a predominant role during activation through its inhibition of productive interactions between two other essential oxidase components. These findings again illustrate the central role played by multiple SH3 domain interactions in modulation of NADPH oxidase activity. In this case, we demonstrated down-regulation by an accessory SH3 domain-containing protein (p40phox), which by virtue of its homology to one essential oxidase component (p67phox) competes for a common target site on another. This SH3 target site in p47phox is flanked by sites that were recently shown to be phosphorylated during oxidase activation (35). Future work will examine whether phosphorylation alters binding affinities of these competing SH3 domains and whether these changes are critical in the activation process.

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