Activation of the Phagocyte NADPH Oxidase Protein p47phox

PHOSPHORYLATION CONTROLS SH3 DOMAIN-DEPENDENT BINDING TO p22phox

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Activation of phagocyte NADPH oxidase requires interaction between p47phox and p22phox. p47phox in resting phagocytes does not bind p22phox. Phosphorylation of serines in the p47phox C terminus enables binding to the p22phox C terminus by inducing a conformational change in p47phox that unmask the SH3α domain. We report that an arginine/lysine-rich region in the p47phox C terminus binds the p47phox SH3 domains expressed in tandem (SH3αβ) but does not bind the individual N-terminal SH3α and C-terminal SH3β domains. Peptides matching amino acids 301–320 and 314–335 of the p47phox arginine/lysine-rich region block the p47phox SH3αβ/p22phox C-terminal and p47phox SH3αβ/p47phox C-terminal binding and inhibit NADPH oxidase activity in vitro. Peptides with phosphoserines substituted for serines 310 and 328 do not block binding and are poor inhibitors of oxidase activity. Mutated full-length p47phox with aspartic acid substitutions to mimic the effects of phosphorylations at serines 310 and 328 bind the p22phox proline-rich region in contrast to wild-type p47phox. We conclude that the p47phox SH3αβ domain-binding site is blocked by an interaction between the p47phox SH3αβ domains and the C-terminal arginine/lysine-rich region. Phosphorylation of serines in the p47phox C terminus disrupts this interaction leading to exposure of the SH3α domain, binding to p22phox, and activation of the NADPH oxidase.

The NADPH oxidase complex found primarily in neutrophils and other myeloid phagocytes is a critical component of microbicidal defenses against bacterial, fungal, and viral pathogens. NADPH oxidase catalyzes reduction of molecular oxygen to superoxide with electrons derived from NADPH (1). Superoxide itself is a weak oxidant but is converted by enzymatic and nonenzymatic pathways to more potent oxidants including hydrogen peroxide, hydroxyl radical, hypohalous acids, and other reactive oxygen species (2). Neutrophils localize NADPH oxidase to the phagolysosomal membrane surrounding engulfling microbes, which in theory limits oxidation of host proteins, lips, and nucleic acids. It is not surprising that formation of the NADPH oxidase is controlled precisely to minimize inappropriate activation of superoxide generation in the absence of infection (1, 3).

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The catalytic core of NADPH oxidase is the integral membrane protein cytochrome b558 that consists of equimolar amounts of gp91phox and p22phox subunits in a 1:1 complex (4, 5). Cytochrome b558 binds NADPH and FAD as well as two hemes (6, 7). However, cytochrome b558 is inactive in the absence of at least three cytosol proteins p47phox, p67phox, and Rac1 (or Rac2) that translocate to membrane and bind cytochrome b558 coincident with stimulation of neutrophil pathways that elicit generation of superoxide (3, 6, 7). The functions of the cytosolic components are not completely understood, but p47phox is required to facilitate electron transfer via cytochrome b558 (8, 9). Rac1 and Rac2, members of the Rho class GTPases, exist in cytosol in the inactive GDP-bound state. Stimulation of neutrophils leads to GTP/GDP exchange, and the resulting active GTP-bound Rac binds p67phox a step required for NADPH oxidase activation (10–12). Stimulation of neutrophils with phorbol myristate acetate leads to multiple phosphorylations of p47phox serines by one or more kinases with the most heavily phosphorylated forms of p47phox bound to membrane (13–16). p47phox can be activated in a cell-free NADPH oxidase system by in vitro phosphorylation with protein kinase C (17).

There are two SH3β domains located in the middle of p47phox. The N-terminal SH3 domain (SH3α) binds to a proline-rich region in the p22phox C terminus, an interaction essential for NADPH oxidase activity (18, 19). The function of the C-terminal SH3 domain (SH3β) is less well understood. A proline-rich region conforming to a consensus SH3 domain-binding site is found at the p47phox C terminus. Potential targets for the p47phox proline-rich region include SH3 domains in p67phox, p40phox, and p47phox (18–26). A positively charged arginine- and lysine-rich (Arg/Lys) domain is located in the p47phox C terminus between the SH3α domain and the C-terminal proline-rich sequence. Multiple serines are found in this Arg/Lys domain with amino acid sequences conforming to consensus phosphorylation sites recognized by several protein kinases (16). The Arg/Lys domain and C-terminal proline-rich domain are thought to regulate NADPH oxidase assembly but are not required for NADPH oxidase catalytic activity since a recombinant truncated p47phox lacking these two domains is fully functional (27).

Babior and collaborators (15, 16) showed that most p47phox serines phosphorylated in response to phorbol stimulation of neutrophils mapped to the Arg/Lys domain. To test the contributions of phosphorylations of individual serines to overall p47phox activity, Faust et al. (28) constructed a series of p47phox mutants with alanine substitutions for every individual p47phox serine between residues 303 and 379. EBV-trans-...
Phosphorylation Controls p47\textsubscript{phox} SH3 Domain Binding to p22\textsubscript{phox}

**RESULTS**

Full-length p47\textsubscript{phox} does not bind spontaneously to cytochrome b\textsubscript{558} in the absence of activation despite affinity of the p47\textsubscript{phox} N-terminal SH3\textsubscript{A} domain for the p22\textsubscript{phox} C-terminal protein-rich region (21, 34). EGY48 expressing full-length p47\textsubscript{phox}/B42 ad and the p22\textsubscript{phox} C-terminal/LexA bd did not grow on leucine-deficient media (see below) in contrast to yeast expressing p47\textsubscript{phox}/SH3AB/or p47\textsubscript{phox}/SH3A/B42 ad with the p22\textsubscript{phox} C-terminal/LexA bd (Fig. 2a). We hypothesized that access to the SH3\textsubscript{A} domain is blocked in full-length p47\textsubscript{phox} by another region of p47\textsubscript{phox} that obstructs the SH3\textsubscript{A} domain binding groove. The abilities of N-terminal and C-terminal p47\textsubscript{phox} truncations to bind to the p47\textsubscript{phox} SH3 domains were tested in the yeast two-hybrid assay (Fig. 2b). p47\textsubscript{phox} truncations containing the C-terminal Arg/Lys-rich domain p47\textsubscript{phox}/SH3AB, p47P, which encompasses the p47\textsubscript{phox} C-terminal protein-rich peptide, bound p47S33A but not p47S33AB.

We investigated which regions within the p47\textsubscript{phox} Arg/Lys domain bound the SH3\textsubscript{A}BD domains. Several laboratories reported that synthetic peptides corresponding to Arg/Lys domain sequences inhibited NADPH oxidase activity in vitro.

**Fig. 1.** Schematic showing p47\textsubscript{phox} truncations used in these experiments. N termini of full-length and truncated p47\textsubscript{phox} are on the left. Shown are names and amino acid spans for the p47\textsubscript{phox} SH3 domains, a solid vertical bar, + + + denotes approximate position of the arginine/lysine-rich domain in the p47\textsubscript{phox} C terminus. Sequences and positions of synthetic p47\textsubscript{phox}-(301–320)- and (314–325)-peptides within the Arg/Lys domain are shown (serines 310 and 328 in bold).

**MATERIALS AND METHODS**

*Yeast Two-hybrid Binding Assay—Saccharomyces cerevisiae* EGY48, pEG202, pJG4-5, and pSH18-34 were generous gifts of Roger Brent (Massachusetts General Hospital, Boston) and Erica Golemis (Fox Chase Cancer Center, Philadelphia) (29). cDNA encoding p22\textsubscript{phox} C terminus (residues 135–195), p22\textsubscript{phox} C terminus (residues 135–195), P156Q (concentration of peptide that inhibited 50% of NADPH oxidase activity) was calculated as described (30). Recombinant p47NT-SH3AB (residues 1–285) and RalC185S were expressed in DH5a *Escherichia coli* K12 strain SH3AB, p47NT SH3AB were cleaved from GST with thrombin as described (31). Recombinant full-length p47\textsubscript{phox} and p67\textsubscript{phox} were expressed in baculovirus-infected S9 cells and purified as described (32).

**Affinity Precipitation and Immunoblot Studies—Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) (20 µl) bound with GST-p22\textsubscript{CT}, GST-p22\textsubscript{p22\textsubscript{CT}}, and p47CT were washed and resuspended in 250 µl of phosphate-buffered saline (157 mM NaCl, 2.7 mM KCl, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate, pH 7.4) containing 0.2% Tween 20. Synthetic peptides and p47NT SH3AB were added, and the final volume was adjusted to 500 µl with deionized water. Final concentrations of peptides and p47\textsubscript{phox} SH3AB were 250 µM and 1.5 µM, respectively. The mixture was gently tumbled at room temperature for 2 h and washed three times with 2-fold diluted phosphate-buffered saline containing 0.2% Tween 20. The beads were pelleted, resuspended in 50 µl of Laemmli sample buffer (33), and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 4–20% Tris glycine gels (NOVEX, San Diego, CA) and transferred onto Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5) for 1 h at room temperature. Membranes were incubated with 1:2500 goat anti-p47\textsubscript{phox} antibody for 1 h at room temperature, washed extensively, and incubated with phosphatase-conjugated 1:5000 rabbit anti-goat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium phosphate substrate (Kirkegaard & Perry Laboratories).

**RESULTS**

Expression of LexA and B42 fusion proteins in EGY48 was confirmed by immunoblot analysis (12). Proteolytic processing in leucine-deficient media indicated reconstitution of LexA transactivator function and transcription of the LEU2 reporter (29).

**Cell-free Assay for NADPH Oxidase Activity—Neutrophil membrane and cytosol fractions were prepared as described (30). Peptides were synthesized by the Biopolymer Laboratory at the University of Maryland, Baltimore. Final peptide purity was >90% estimated by high pressure liquid chromatography. Correct sequence of synthesized peptides was confirmed by mass spectrometry.**
p22CT binding by p47
phoserylserine for serine 310 decreased inhibition of p47SH3AB/Phosphoserine substitutions at serines 315, 320, and 328 did p22CT (Fig. 4). A similar Arg/Lys domain peptide with three S328pS, containing a single phosphoserine substitution for serines. Similarly, phosphorylated p47SH3AB and p22CT is SH3 domain-dependent since no binding to p22CT is seen (P156Q mutation eliminates affinity of the p22box proline-rich region for the p47box SH3 domain) (21, 34). Figure shown is representative of at least five similar experiments.

We synthesized peptides corresponding to Arg/Lys domain sequences encompassing p47phox serines 310 and 328. Immobilized GST-p47phox C terminus (p47CT) consisting of the Arg/Lys domain plus the C-terminal proline-rich region was incubated with p47phox SH3 AB domains (p47SH3AB) with and without p47phox peptides-(301–320) and -(314–335) (Fig. 3). Both peptides blocked co-precipitation of p47SH3AB by GST-p47CT. p47phox-peptide-(358–365), corresponding to the p47phox C-terminal proline-rich region, did not block co-precipitation of p47SH3AB by GST-p47CT. Both Arg/Lys domain peptides also blocked co-precipitation of p47SH3AB by GST-p22CT (Fig. 4) even though the p47phox Arg/Lys domain does not bind the p22box C terminus in yeast two-hybrid or affinity precipitation experiments (data not shown). p47phox Arg/Lys-(314–335)-peptide was equivalent to p22phox-(149–165), a peptide corresponding to the p22phox proline-rich sequence, which would be expected to be a competitive inhibitor of p47SH3AB/p22CT binding (Fig. 4b).

Additional p47phox Arg/Lys-(301–320)- and -(314–335)-peptides with phosphoserines substituted for serines 310 and 328 were used to assess the effects of phosphorylation of serines 310 and 328 on p47phox Arg/Lys domain function. In contrast to p47phox Arg/Lys-(314–335), p47phox Arg/Lys-(314–335)-S328pS, containing a single phosphoserine substitution for serine 328, did not inhibit co-precipitation of p47SH3AB by GST-p22CT (Fig. 4b). A similar Arg/Lys domain peptide with three phosphoserine substitutions at serines 315, 320, and 328 did not inhibit p47SH3AB/p22CT binding. Substitution of phosphoserine for serine 310 decreased inhibition of p47SH3AB/p22CT binding by p47phox Arg/Lys-(301–320)-S310pS compared with wild-type Arg/Lys-(301–320)-peptide (Fig. 4a). Similarly, phosphorylated p47phox Arg/Lys-(301–320)- and -(314–335)-peptides were less potent inhibitors of p47SH3AB/p47CT binding compared with wild-type Arg/Lys peptides (Fig. 3).

We examined the effects of p47phox serines 310 and 328 on p47phox binding to the p22phox C terminus in the yeast two-hybrid assay (Fig. 5). Mutations substituting alanines and aspartic acids at serines 310 and 328 were introduced into p47phox DNA. Alanine substitutions were used to assess the effects of serine loss since alanine presents a steric profile similar to serine but has neutral polarity and cannot form hydrogen bonds. Aspartic acid substitutions mimic the negative charges that occur with phosphorylations of serines. In contrast to wild-type p47phox, both p47phox S310A and p47phox S310D/B42 ad interacted with p22phox C-terminal/LexA bd in the two-hybrid assay (Fig. 5). Binding of full-length p47phox S328A to the p22phox C terminus was not substantially different from wild-type p47phox. However, the S328D mutation enabled p47phox/p22phox C-terminal binding. The p47phox S310D/S328D double mutant also bound p22phox C terminus. Substituted p47phox/p22phox C-terminal binding was SH3 domain-dependent since no binding was seen to p22phox P156Q C terminus. Serine 310 and 328 substitutions did not affect binding of the p47phox C-terminal proline-rich region to the p67phox-C-terminal SH3B.

The effects of p47phox Arg/Lys domain peptides-(301–320) and -(314–335) on NADPH oxidase activity were tested in the cell-free assay (Fig. 6) using membrane and cytosol fractions isolated from resting neutrophils. Levels of NADPH oxidase activity were quantitated as maximal rates of superoxide generation (Vmax) expressed as milliOD/min (Fig. 6). Vmax was measured with varying concentrations of wild-type and phosphorylated p47phox Arg/Lys domain peptides. Wild-type p47phox-(301–320)- and -(314–335)-peptides inhibited superoxide production in a dose-dependent manner with IC50 values among the lowest reported for peptide inhibitors of NADPH oxidase activation (Table I). Phosphorylated p47phox Arg/Lys peptides were 7–20-fold less potent inhibitors of NADPH oxidase activity compared with wild-type peptides (Table I).

The role of the p47phox Arg/Lys domain in activating the NADPH oxidase was examined further in a cell-free system with recombinant p67phox, Rac1 C189S, and p47NT-SH3AB (instead of full-length p47phox) used in place of neutrophil cytosol. p47NT-SH3AB spanning amino acids 1–285 is a truncated p47phox consisting of the N terminus plus both SH3
Phosphorylation Controls \(p47^{\text{phox}}\) SH3 Domain Binding to \(p22^{\text{phox}}\)

**Fig. 4.** \(p47^{\text{phox}}\)-Arg/Lys-(301–320)- and -(314–335)-peptides block binding of \(p47^{\text{SH3AB}}\) to \(p22^{\text{CT}}\) by immunoblot analysis. GST- \(p22^{\text{CT}}\) immobilized on glutathione-agarose beads was incubated with \(p47^{\text{SH3AB}}\) with and without native and phosphorylated Arg/Lys-(301–320)-peptides (a) and -(314–335)-peptides (b) as described under “Materials and Methods.” GST- \(p22^{\text{CT}}\) was substituted for GST- \(p22^{\text{CT}}\) in the control lane. Immobilized proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and incubated with anti- \(p47^{\text{phox}}\) serum. Scrambled peptides corresponding to Arg/Lys-(301–320) and -(314–335) were substantially less potent in their abilities to block co-precipitation of \(p47^{\text{SH3AB}}\) by GST- \(p22^{\text{CT}}\) compared with native sequence peptides (data not shown). Figure shown is representative of three experiments.

**Table I**

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>IC(_{50}) (μM)</th>
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<tbody>
<tr>
<td>(p47^{\text{phox}})-(301–320)</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>(p47^{\text{phox}})-(301–320) S10pS</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>(p47^{\text{phox}})-(306–325)</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>(p47^{\text{phox}})-(314–335)</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>(p47^{\text{phox}})-(314–335) S328pS</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>(p47^{\text{phox}})-(314–335) S315,320,328pS</td>
<td>130 ± 9</td>
</tr>
</tbody>
</table>

**Fig. 5.** Mutations at \(p47^{\text{phox}}\) serines 310 and 328 unmask the SH3 domains and enable \(p47^{\text{phox}}/p22^{\text{phox}}\) binding by yeast two-hybrid analysis. Alanine and aspartic acid substitutions for serines 310 and 328 were introduced into full-length \(p47^{\text{phox}}\) using site-directed mutagenesis methods as described under “Materials and Methods.” Yeast expressing either native or substituted full-length \(p47^{\text{phox}}\) fused to B42 ad with either \(p22^{\text{CT}}\), \(p22^{*}\text{CT}\), or \(p67\) fused to LexA bd were spotted on leucine-deficient media. Alanine and aspartic acid substitutions at serines 310 and 328 did not affect \(p47^{\text{phox}}/p67\) binding. Binding of \(p47^{\text{phox}}\) S310A, \(p47^{\text{phox}}\) S310D, \(p47^{\text{phox}}\) S328D, and \(p47^{\text{phox}}\) S310D S328D to \(p22^{\text{CT}}\) was SH3 domain-dependent since no binding to \(p22^{\text{CT}}\) was seen. Figure shown is representative of five experiments.

**Fig. 6.** Phosphorylation diminishes inhibition of NADPH oxidase activity by the \(p47^{\text{phox}}\) Arg/Lys domain-(314–335)-peptide in the cell-free assay. Neutrophil membrane and cytosol fractions were incubated with GTP-\(S\), NADPH, and increasing concentrations of \(p47^{\text{phox}}\) ( ), \(p47^{\text{phox}}\) Arg/Lys-(314–335) ( ), and \(p47^{\text{phox}}\) Arg/Lys-(301–320) ( ) with arachidonic acid to initiate NADPH oxidase activity (see “Materials and Methods”). Maximal rates of superoxide generation (\(V_{\text{max}}\)) were determined as described under “Materials and Methods.” Each point is the mean of duplicate wells, and plots shown are representative of three experiments.

for activating the oxidase in vitro. Superoxide generation in vitro using \(p47^{\text{NT-SH3AB}}\) was equivalent with and without arachidonic acid (Fig. 7). In contrast to \(p47^{\text{NT-SH3AB}}\), full-length \(p47^{\text{phox}}\) was completely inactive without arachidonic acid (Fig. 7b). The abilities of \(p47^{\text{phox}}\) Arg/Lys domain-(301–320)- and -(314–335)-peptides to inhibit this \(p47^{\text{NT-SH3AB}}\)-containing cell-free system was not dependent on arachidonic acid. These results indicate that the abilities of Arg/Lys domain peptides to inhibit oxidase activity in vitro do not require the \(p47^{\text{phox}}\) Arg/Lys domain. This suggests that these peptides do not inhibit oxidase activity by interfering with some function of the Arg/Lys domain in full-length \(p47^{\text{phox}}\).

DISCUSSION

NADPH oxidase function requires activation of dormant \(p47^{\text{phox}}\) by phosphorylation of one or more serines clustered in the Arg/Lys domain in the C terminus. Considerable insight into \(p47^{\text{phox}}\) function has been gained in the last few years. \(p47^{\text{phox}}\) is not absolutely required for NADPH oxidase activity in vitro. Normal amounts of superoxide are generated in the cell-free system using neutrophil membrane containing cytochrome \(b_558\), GTP-bound Rac, and very high concentrations of \(p67^{\text{phox}}\) (8, 9). \(p47^{\text{phox}}\) functions in neutrophils as an adaptor protein that facilitates binding of other oxidase components to each other. \(p47^{\text{phox}}\) has been reported to bind \(p67^{\text{phox}}\), \(p40^{\text{phox}}\), and \(p22^{\text{phox}}\) mediated by SH3 domain-dependent interactions and to bind also to \(p91^{\text{phox}}\) (18–27, 34, 37, 40). \(p47^{\text{phox}}\) does not translocate spontaneously to membrane in resting neutrophils (41), and \(p47^{\text{phox}}\) does not bind recombinant \(p22^{\text{phox}}\) C terminus in vitro without arachidonic acid (34). These observations suggest that access by the \(p22^{\text{phox}}\) proline-rich region to the \(p4^{\text{phox}}\) SH3 domain is blocked in unphosphorylated \(p47^{\text{phox}}\). The requirement for arachidonic acid or SDS in the cell-free system is obviated by using \(p47^{\text{phox}}\) phosphorylated in vitro by protein kinase C (17). Exogenous phos-
Phosphorylation by protein kinase C and incubation with arachidonic acid both induce similar changes in p47phox conformation as measured by changes in tryptophan fluorescence (42) and susceptibility of cysteine 378 to reaction with N-ethylmaleimide (43). Several authors (3, 34, 43) have speculated that conformational changes induced by phosphorylation and anionic amphiphiles arise from rearrangements of internal p47phox binding sites. The presence of cysteine 378 within internal binding sites is suggested by studies with reduced and alkylated p47phox, which reveals increased thiol reactivity in p47phox C terminus peptides and increased accessibility of tryptophan residues in the SH3 domains (42, 43). In vitro treatment with reducing agents reduced the accessibility of tryptophan residues in the SH3 domains even further. These results are consistent with perturbation of internal p47phox binding sites in the presence of arachidonic acid.

In this study, the effects of phosphorylation were examined directly using synthetic p47phox Arg/Lys domain peptides where serines 310 and 328 were replaced by phosphoserines. Phosphoserine-substituted peptides were uniformly less able than wild-type Arg/Lys-(301–320) (314–335) to disrupt binding to the SH3 domain. Phosphorylated Arg/Lys peptides-(301–320) and -(314–335) to disrupt superoxide generation. Phosphoserines cannot be substituted directly for p47phox serines 310 and 328 in the yeast two-hybrid binding experiments. Instead, we examined the effects of alanine and aspartic acid substitutions at serines 310 and 328 in full-length p47phox mutants. Analysis of these substitutions in full-length p47phox overcomes problems with using truncated proteins in binding assays where some aspects of biological specificity may be lost (3). The SH3 domains in p47phox S310D and p47phox S328D both bind p22phox proline-rich regions in the two-hybrid assay suggesting that introducing a single negative charge at either of these two positions is sufficient to disrupt p47phox Arg/Lys domain binding to the SH3AB domains. Alanine substitution at serines 310 and 328 yielded different results. p47phox S328A did not bind to the SH3AB domains indicating that loss of the serine 328 hydroxyl group was not sufficient to disrupt Arg/Lys domain binding to the SH3AB domains. In contrast, p47phox S310A interacted with the SH3AB domains in the two-hybrid assay. This suggests that the serine 310 hydroxyl side group may participate directly in an interaction that stabilizes Arg/Lys domain to the SH3AB domain.

The ability of Arg/Lys peptides-(301–320) and -(314–335) to block co-precipitation of p47SH3AB by GST-p22CT was surprising since the p47phox Arg/Lys domain does not bind p22phox (data not shown) and does not bind to the binding groove in the p47phox SH3 domain (Fig. 2a). P47SH3AB is equivalent to p47SH3A in its ability to bind the p22phox proline-rich region (Fig. 2a) suggesting that p47SH3AB exists normally in an open conformation where access to the p47phox SH3 domain is impeded. Masking the SH3 domain within full-length p47phox involves adoption of a new conformation by the two p47phox SH3 domains in concert such that the binding groove of the SH3 domain is no longer accessible. Binding to the Arg/Lys domain locks p47phox SH3A in this closed conformation (Fig. 8). Stimulation of phagocytes leads to phosphorylation of serines within the p47phox C terminus and disruption of this internal interaction. Phosphorylated p47phox then relaxes into the open conformation characteristic of p47SH3AB with unmasking of the SH3 domain, binding to p22phox, and activation of the NADPH oxidase.

Internal binding of the p47phox Arg/Lys domain to the p47phox SH3A domain appears to be sufficient to block spontaneous activation of the NADPH oxidase in the cell-free system. The
requirement for arachidonic acid as the exogenous activator was bypassed when truncated N-terminal p47\textsuperscript{phox} lacking the Arg/Lys and proline-rich domains was substituted for full-length p47\textsuperscript{phox} (Fig. 7). Inhibition of superoxide generation by p47\textsuperscript{phox} Arg/Lys-(301–320) and -(314–335) was also not dependent on arachidonic acid activation since both peptides inhibited enzyme activity in the cell-free system without arachidonic acid. p47\textsuperscript{phox} SH3\textsubscript{AB} domain binding to the Arg/Lys domain was not dependent on other loci within the p47\textsuperscript{phox} C terminus (such as the p47\textsuperscript{phox} proline-rich region) since Arg/Lys peptides-(301–320) and -(314–335) inhibited oxidase activity with p47NT-SH3AB.

An alternative model for function of the p47\textsuperscript{phox} Arg/Lys domain is that phosphorylation of one or more serines creates a ligand recognized by a binding site on another Phox protein. This would be analogous to how phosphorytrosines are recognized by SH2 domains. Our findings that phosphoserine-containing Arg/Lys peptides are uniformly less potent than native peptides in binding and cell-free oxidase activity experiments argue against this model. A more complex model was presented by DeLeo et al. (36, 37) who hypothesized that the p47\textsuperscript{phox} Arg/Lys domain bound to specific sites in both p67\textsuperscript{phox} and to cytochrome b\textsubscript{5} at different stages of NADPH oxidase assembly. They found several peptides with sequences similar to Arg/Lys domain amino acids compassing serine 328 using purified cytochrome b\textsubscript{5}S and recombinant p67\textsuperscript{phox} as baits in screens of a phage display library. p47\textsuperscript{phox} Arg/Lys peptides inhibited p47\textsuperscript{phox} phosphorylation, translocation of p47\textsuperscript{phox} to membrane in vitro, and superoxide generation in vitro and in permeabilized neutrophils. These experimental findings are consistent with our findings. The fact that we and others (27) can reconstitute NADPH oxidase activity in vitro using p47NT-SH3AB lacking the Arg/Lys domain indicates that the Arg/Lys domain is not required absolutely for activity in the cell-free system. Arg/Lys peptides inhibited oxidase activity in the cell-free system with p47NT-SH3AB indicating that the peptides are not acting as competitive inhibitors of the native p47\textsuperscript{phox} Arg/Lys domain. Rather, Arg/Lys domain peptides mimic the p47\textsuperscript{phox} Arg/Lys domain in vitro. In our model (Fig. 8), the p47\textsuperscript{phox} Arg/Lys domain functions normally to inhibit activation of the oxidase. Therefore, peptides that mimic activity of the Arg/Lys domain also inhibit the oxidase. We anticipate that the situation in cells will be more complicated and that transfection studies using mutated full-length p47\textsuperscript{phox} will prove useful in furthering understanding of p47\textsuperscript{phox} function.
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