Activation of the Phagocyte NADPH Oxidase Protein p47phox

PHOSPHORYLATION CONTROLS SH3 DOMAIN-DEPENDENT BINDING TO p22phox

(Received for publication, January 28, 1999, and in revised form, April 1, 1999)

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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 unmaskss the SH3 domain. We report that an arginine/lysine-rich region in the p47phox C terminus binds the p47phox SH3 domains expressed in tandem (SH3A) and does not bind the individual N-terminal SH3 A and C-terminal SH3 B domains. Peptides matching amino acids 301-320 and 314-335 of the p47phox arginine/lysine-rich region block the p47phox SH3A/p22phox C-terminal and p47phox SH3A/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 SH3 A domain-binding site is blocked by an interaction between the p47phox SH3A 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 SH3 A 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 engulfed microbes, which in theory limits oxidation of host proteins, lipids, 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).

* This work was supported by a Department of Veterans Affairs Merit Review grant and National Institutes of Health Grants AI32220 and AI40007. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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 cytosol components are not completely understood, but p67phox 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 SH3 domains located in the middle of p47phox. The N-terminal SH3 domain (SH3A) 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 (SH3B) 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 SH3A 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 serine phosphorylations 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-
formed B lymphocytes derived from a patient with the p47\textsubscript{phox} deficient form of chronic granulomatous disease were transfected with the alanine-substituted p47\textsubscript{phox} cDNA. No superoxide was generated by p47\textsuperscript{S303A}, p47\textsuperscript{S304A}, or p47\textsuperscript{S310A} expressing B cells expressing p47\textsubscript{phox} and stimulated with phorbol esters, EBV B cells expressing p47\textsubscript{S303A} and p47\textsubscript{S304A} with S303A and S304A, and S328A mutations generated less than 50% of wild-type NADPH oxidase activity. p47\textsubscript{S315A}, p47\textsubscript{S345A}, p47\textsubscript{S348A}, and S328A mutations generated less than 50% of wild-type NADPH oxidase activity. p47\textsubscript{S370A} supported wild-type levels of NADPH oxidase activity, whereas S331A, S345A, S348A, and S370A mutations resulted in only moderate decreases in superoxide generation. In contrast, alanine substitutions at any serine within the Arg/Lys domain activates p47\textsubscript{phox} oxidase activity. p47\textsubscript{S379A} stimulated with phorbol esters. EBV

Superoxide was generated by p47\textsuperscript{S379A} (Fig. 1) and p47\textsuperscript{S392A} (see Fig. 1), and full-length p47\textsubscript{phox}-(301–320)- and -(314–325)-peptides within the Arg/Lys domain are shown (serines 310 and 328 in bold).

**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\textsubscript{A} 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{SH3A}, p47\textsubscript{SH3B}, which encompasses the p47\textsubscript{phox} C-terminal proline-rich peptide, bound p47\textsubscript{SH3A} but not p47\textsubscript{SH3AB}. We investigated which regions within the p47\textsubscript{phox} Arg/Lys domain bound the SH3\textsubscript{A} domains. Several laboratories reported that synthetic peptides corresponding to Arg/Lys domain sequences inhibited NADPH oxidase activity in vitro.
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 p47SH3AB 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-(358–385), 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 p22phox 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).

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 \( V_{max} \). NADPH oxidase activity were quantitated as maximal rates of superoxide generation \( V_{max} \). 

![Diagram](image.png)
Phosphorylation Controls p47phox SH3 Domain Binding to p22phox

**FIG. 4.** p47phox Arg/Lys-(301–320)- and -(314–335)-peptides block binding of p47SH3AB to p22CT by immunoblot analysis. GST-p22CT immobilized on glutathione-agarose beads was incubated with p47SH3AB with and without native and phosphorylated Arg/Lys-(301–320)-peptides (a) and -(314–335)-peptides (b) as described under "Material and Methods." GST-p22CT was substituted for GST-p22CT in the control lane. Immobilized proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and incubated with anti-p47phox serum. Scrambled peptides corresponding to Arg/Lys-(301–320) and -(314–335) were substantially less potent in their abilities to block co-precipitation of p47SH3AB by GST-p22CT compared with native sequence peptides (data not shown). Figure shown is representative of three experiments.

**FIG. 5.** Mutations at p47phox serines 310 and 328 unmask the SH3 domains and enable p47phox/p22phox binding by yeast two-hybrid analysis. Alanine and aspartic acid substitutions for serines 310 and 328 were introduced into full-length p47phox using site-directed mutagenesis methods as described under "Materials and Methods." Yeast expressing either native or substituted full-length p47phox fused to B42 ad with either p22CT, p22CT, 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 p47phox/p67phox binding. Binding of p47phox S310A, p47phox S310D, p47phox S310P, p47phox S310R, p47phox S310T, p47phox S310V, and p47phox S310W to p22CT was SH3 domain-dependent since no binding to p22CT was seen. Figure shown is representative of five experiments.

**FIG. 6.** Phosphorylation diminishes inhibition of NADPH oxidase activity by the p47phox Arg/Lys domain-(314–335)-peptide in the cell-free assay. Neutrophil membrane and cytosol fractions were incubated with GTP-γS, NADPH, and increasing concentrations of p47phox-(314–335) ( ), p47phox-(314–335) S310A ( ), and p47phox-(314–335) S310D ( ) with arachidonic acid to initiate NADPH oxidase activity (see "Materials and Methods"). Maximal rates of superoxide generation (Vmax) were determined as described under "Materials and Methods." Each point is the mean of duplicate wells, and plots shown are representative of three experiments.

**TABLE I**

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>IC50 (mM)</th>
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<tr>
<td>p47phox(301–320)</td>
<td>7.0 ± 0.3</td>
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<tr>
<td>p47phox(301–320) S310pS</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>p47phox(306–325)</td>
<td>25 ± 1</td>
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<tr>
<td>p47phox(314–335)</td>
<td>6.7 ± 0.3</td>
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<tr>
<td>p47phox(314–335) S328pS</td>
<td>44 ± 2</td>
</tr>
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<td>p47phox(314–335) S315,320,328pS</td>
<td>130 ± 9</td>
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**DISCUSSION**

NADPH oxidase function requires activation of dormant p47phox by phosphorylation of one or more serines clustered in the Arg/Lys domain in the C terminus. Considerable insight into p47phox function has been gained in the last few years. p47phox 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 b558, GTP-bound Rac, and very high concentrations of p67phox (8, 9). p47phox functions in neutrophils as an adaptor protein that facilitates binding of other oxidase components to each other. p47phox has been reported to bind p67phox, p40phox, and p22phox mediated by SH3 domain-dependent interactions and to bind also to gp91phox (18–27, 34, 37, 40). p47phox does not translocate spontaneously to membrane in resting neutrophils (41), and p47phox does not bind recombinant p22phox C terminus in vitro without arachidonic acid (34). These observations suggest that access by the p22phox proline-rich region to the p47phox SH3 domain is blocked in unphosphorylated p47phox. The requirement for arachidonic acid or SDS in the cell-free system is obviated by using p47phox 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 site and result in exposure of the p47phox SH3 domain.

We hypothesized that unmasking the p47phox SH3 domain binding site requires disruption of binding between the p47phox C terminus and the two SH3 domains. We found that the p47phox Arg/Lys domain bound p47SH3AB in a survey of potential p47phox-binding sites using the yeast two-hybrid assay. This result was confirmed in affinity precipitation experiments where GST-p47CT co-precipitated p47SH3AB (Fig. 3). The p47phox C terminus contains a proline-rich region in addition to the Arg/Lys domain. Several laboratories identified the p47phox proline-rich region as the ligand for the p67phox C-terminal SH3 domain (20, 21). In the yeast two-hybrid assay, we found that the p47phox proline-rich region bound p47SH3A but not p47SH3B or p47SH3AB. This result is in partial agreement with observations by de Mendez et al. (19) who showed that GST-p47SH3A, but not GST-p47SH3B, bound full-length p47phox. These authors assumed that the p47phox proline-rich region was the ligand for p47phox SH3 domain binding site.

Synthetic Arg/Lys domain peptides-(301–320) and -(314–335) inhibited co-precipitation of p47SH3AB by GST-p47CT in contrast to p47phox proline-rich peptide-(358–385). These two Arg/Lys domain peptides were potent inhibitors of NADPH oxidase activity in vitro (Table I) in contrast to the p47phox proline-rich peptide (data not shown). The p47phox Arg/Lys domain is the critical determinant for p47phox C-terminal binding to the p47phox SH3 domain. The p47phox proline-rich region failed to bind p47PHAS SH3AB in the yeast two-hybrid experiments, and the p47phox proline-rich region peptide did not block p47CT C-terminal/SH3AB binding nor inhibit superoxide production. This result is not surprising; if phosphorylation toggles the p47phox activation switch, then the Arg/Lys domain that encompasses most of the phosphorylated p47phox serines should be intimately involved in p47phox internal binding.

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) and -(314–335) peptides to block co-precipitation of p47SHAB by GST-p22CT and GST-p47CT and to inhibit cell-free 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 SH3A domains in p47phox S310D and p47phox S328D both bound 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. Alamine 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 abilities 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 to 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 unimpeded. Masking the SH3A 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 SH3A 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 SH3A 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 p47phox lacking the Arg/Lys and proline-rich domains was substituted for full-length p47phox (Fig. 7). Inhibition of superoxide generation by p47phox Arg/Lys-(301–320) and -(314–335) was also not dependent on arachidonic acid activation since both peptides inhibited oxidase activity in the cell-free system without arachidonic acid. p47phox SH3AB domain binding to the Arg/Lys domain is not dependent on other loci within the p47phox C terminus (such as the p47phox 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 p47phox 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 phosphorytosynes 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 p47phox Arg/Lys domain bound to specific sites in both p67phox and to cytochrome b556 at different stages of NADPH oxidase assembly. They found several peptides with sequences similar to Arg/Lys domain amino acidscompassing serine 328 using purified cytochrome b556 and recombinant p67phox as baits in screens of a phage display library. p47phox Arg/Lys peptides inhibited p47phox phosphorylation, translocation of p47phox 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 p47phox Arg/Lys domain. Rather, Arg/Lys domain peptides mimic the p47phox Arg/Lys domain in vitro. In our model (Fig. 8), the p47phox 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 p47phox will prove useful in furthering understanding of p47phox function.

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