Assembly of the Human Neutrophil NADPH Oxidase Involves Binding of p67\textsuperscript{phox} and Flavocytochrome b to a Common Functional Domain in p47\textsuperscript{phox*}

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The human neutrophil NADPH oxidase is a multi-component complex composed of membrane-bound and cytosolic proteins. During activation, cytosolic proteins p47\textsuperscript{phox}, p67\textsuperscript{phox}, Rac2, and possibly p40\textsuperscript{phox} translocate to the plasma membrane and associate with flavocytochrome b to form the active superoxide-generating system. To further investigate the role of p67\textsuperscript{phox} in this complex assembly process, experiments were performed to identify possible regions of interaction between p67\textsuperscript{phox} and other NADPH oxidase proteins. Using random sequence peptide phage-display library analysis of p67\textsuperscript{phox}, we identified a novel region in p47\textsuperscript{phox} encompassing residues 323–332 and a previously identified SH3 binding domain encompassing p47\textsuperscript{phox} residues 361–370 as p67\textsuperscript{phox} binding sites. Synthetic peptides mimicking p47\textsuperscript{phox} residues 323–332 inhibited the p47\textsuperscript{phox}-p67\textsuperscript{phox} binding interaction in an affinity binding assay; however, peptides mimicking flanking regions were inactive. Surprisingly, this same region of p47\textsuperscript{phox} was found previously to represent a site of binding interaction for flavocytochrome b (DeLeo, F. R., Nauseef, W. M., Jesaitis, A. J., Burritt, J. B., Clark, R. A., and Quinn, M. T. (1995) J. Biol. Chem. 270, 26246–26251), and this observation was confirmed in the present report using two different in vitro assays that were not evaluated previously. Using affinity binding assays, we also found that p67\textsuperscript{phox} and flavocytochrome b competed for binding to p47\textsuperscript{phox} after activation, suggesting that prior to full NADPH oxidase assembly the 323–332 region of p47\textsuperscript{phox} is associated with p67\textsuperscript{phox} and at some point in the activation process is transferred to flavocytochrome b. Thus, taken together our data demonstrate that both p67\textsuperscript{phox} and flavocytochrome b utilize a common binding site in p47\textsuperscript{phox} presumably at distinct stages during the activation process, and this p47\textsuperscript{phox} region plays a key role in regulating NADPH oxidase assembly.

Human neutrophils play an essential role in the body's defense against invasion of pathogenic organisms and are also one of the primary cell types involved in the inflammatory response. Upon exposure to a pathogenic stimulus, the neutrophil becomes activated and generates superoxide anion (O2\textsuperscript{-}) and other more potent antimicrobial agents (1, 2). The generation of these oxidants by neutrophils occurs through the activation of a membrane-associated complex known as the NADPH oxidase. In resting cells, the components of the NADPH oxidase are segregated into cytosolic and membrane compartments; during activation, however, the cytosolic components translocate to the plasma membrane and assemble with membrane-bound components, resulting in the active, O2\textsuperscript{-}-generating system (2–5).

The key membrane-associate component of the NADPH oxidase is a heterodimeric flavocytochrome b which is composed of a 91-kDa glycoprotein (gp91\textsuperscript{phox}) and a 22-kDa protein (p22\textsuperscript{phox}) (6–8). Flavocytochrome b has been reported to contain all of the redox components of the NADPH oxidase (7–9) and is the key catalytic component responsible for the direct transfer of electrons from NADPH to molecular oxygen. In neutrophil membranes, a low molecular weight GTP-binding protein, Rap1A, is also associated with flavocytochrome b and plays a role in NADPH oxidase regulation in vivo (10–12). The essential cytosolic components of the NADPH oxidase are known as p47\textsuperscript{phox}, p67\textsuperscript{phox}, and a second low molecular weight GTP-binding protein, Rac (13–17). All three of these cytosolic proteins have been shown to translocate from the cytosol to the membrane during NADPH oxidase assembly (1, 3–5). A fourth cytosolic protein known as p40\textsuperscript{phox} has been shown to interact with p47\textsuperscript{phox} and p67\textsuperscript{phox} and may function to stabilize a 240-kDa complex of cytosolic oxidase proteins in resting cells (18, 19).

According to the current model of NADPH oxidase assembly, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and possibly p40\textsuperscript{phox} translocate in an en bloc to associate with flavocytochrome b during neutrophil activation (3, 20). Rac appears to translocate simultaneously but independently of the other cytosolic components to associate with the membrane-bound NADPH oxidase (5, 21, 22). Studies of oxidase assembly in neutrophils from patients with various forms of chronic granulomatous disease suggest that p47\textsuperscript{phox} binds directly to flavocytochrome b (23), and at least six regions of flavocytochrome b have been identified as potential sites for interaction with p47\textsuperscript{phox} (24–29). Complimentary sites of interaction on p47\textsuperscript{phox} have recently been mapped to a cationic domain encompassing residues 323–342 (30) and to the first and possibly second SH3 domain of p47\textsuperscript{phox} (27, 28). However, the process whereby p47\textsuperscript{phox} associates with p67\textsuperscript{phox} to form a complex that translocates to the membrane and the ultimate assembly of the active NADPH oxidase complex is not fully understood. Recently, there have been several reports that SH3 domain interactions mediate the formation of a p47\textsuperscript{phox}-p67\textsuperscript{phox} complex (27, 28, 31, 32) and possibly a complex with p40\textsuperscript{phox} (18, 19); however, the function of these regions in NADPH oxidase activation still remains unclear. Peptides mimicking the SH3 domain target sites are relatively weak...
inhibitors of NADPH oxidase complex formation and have little effect on $O_2^*$ production in the cell-free assay (26, 31, 33). In addition, SH3-mediated interactions between p47$^{phox}$ and p67$^{phox}$ are found in resting neutrophils and in unactivated in vitro assay systems (28, 31, 34–36), suggesting that SH3 domains mediate only part of the assembly process but are not completely responsible for the activation-induced assembly of a stable NADPH oxidase.

To gain a further understanding of the assembly of the NADPH oxidase complex and investigate the interaction of p47$^{phox}$ with p67$^{phox}$, we used peptide mapping in an in vitro binding assay and random sequence peptide phage-display library analysis to define regions of p47$^{phox}$ that interact with p67$^{phox}$. Our data not only confirmed previous reports that the proline-rich carboxyl-terminal domain of p47$^{phox}$ (residues 361–370) is involved in p67$^{phox}$ binding (28, 31, 32, 34, 36) but also demonstrated the presence of a novel binding site for p67$^{phox}$ encompassing residues 323–332 of p47$^{phox}$. Surprisingly, this same region of p47$^{phox}$ also binds to flavocytochrome b (30), and our present studies now provide direct evidence that p47$^{phox}$ 323–332 represents a mutually exclusive binding site for both p67$^{phox}$ and flavocytochrome b and that this p47$^{phox}$ region plays a central role in NADPH oxidase assembly.

**MATERIALS AND METHODS**

Preparation and Fractionation of Neutrophils—Purified human neutrophils, isolated as described previously (37), were disrupted by N$_2$ cavitation by sequential centrifugation as described by Fujita et al. (37).

Production and Purification of Recombinant p67$^{phox}$, p47$^{phox}$, and Rac2—Recombinant p47$^{phox}$ and Rac2—Recombinant p47$^{phox}$ and p67$^{phox}$ were produced in a baculovirus expression system and purified as described by Leto et al. (38). Glutathione S-transferase (GST)-Rac2 was produced in Escherichia coli using a pGEX-2T expression vector (kind gift of Dr. Ulla Knaus, Scripps Research Institute, La Jolla, CA) (39), and GST was cleaved from Rac2 using thrombin (40). All recombinant proteins were found to be $>95\%$ pure using SDS-PAGE and Coomassie Blue staining, their identity was confirmed by Western blotting (data not shown), and they were found to be active in reconstituting NADPH oxidase activity in a cell-free NADPH oxidase assay system containing only recombinant cytosolic proteins (see below). In addition, GTP-binding activity of the recombinant Rac2 was confirmed using an $[^{35}S]$GTP$^\gamma$S binding assay as described by Bokoch and Quilliam (41) (data not shown).

Peptide Synthesis—Peptide RPPGFSPFR was obtained from The American Peptide Co. (Sunnyvale, CA); peptide AYRRNRSVRFL was obtained from Macromolecular Resources (Colorado State University, Fort Collins, CO); and all other peptides were synthesized by the Montana State University peptide synthesis facility. The sequence and purity of all synthetic peptides was confirmed by high performance liquid chromatography and mass spectrometry.

Protein Affinity Binding Assays—Recombinant p47$^{phox}$, p67$^{phox}$, and bovine serum albumin (control) were conjugated to CNBr Sepharose 4B at 1–2 mg of protein/mL of Sepharose following the manufacturer’s instructions (Pharmacia Biotech Inc.). To determine NADPH oxidase protein binding to the conjugated Sepharose beads, 20–50 $\mu$L of beads were incubated with 5–25 $\mu$L of recombinant p67$^{phox}$ or p47$^{phox}$, 4 x 10$^6$ cell equivalents of human neutrophil cytosol or 10$^6$ cell equivalents of human neutrophil membranes in cell-free assay buffer (10 mM sodium phosphate, 130 mM NaCl, 2 mM EGTA, and 2.7 mM KCl (pH 7.2)) containing 10 $\mu$M GTP$^\gamma$S, and 100 $\mu$M SDS in a final volume of 500 $\mu$L). Reactions were incubated with mixing for 3 h at 4°C, pelleted in a microcentrifuge at 8000 rpm for 4 min, and then subsequently washed 4 times in 1 mL of cell-free assay buffer. Samples were then treated identical to Sepharose beads and analyzed by SDS-PAGE and Western blotting to detect bound proteins. Non Specific binding of proteins to the beads was determined using unconjugated and BSA-conjugated Sepharose beads. In all experiments, non specific binding was <5% of the specific binding observed.

| $[^{35}S]$GDPQAVRNYRSVRFL | $^{[35}S]$QPQAVFVPRSSADL |
|----------------------------|----------------------|
| 173 NQDG YAV RNYRSVRFL 183 | 306 KFQVAVFPRSSADL |

1 The abbreviations used are: GST, glutathione S-transferase; PACE, polycyramide gel electrophoresis; BSA, bovine serum albumin; GTP$^\gamma$S, guanosine 5’-O-(thiotriphosphate).
RESULTS

Random Sequence Peptide Phage-Display Library Analysis of p67phox—To identify possible regions of interaction between p67phox and other NADPH oxidase proteins, we screened a random sequence peptide phage-display library with recombinant p67phox to affinity-select phage from the library that specifically bound to p67phox. The predicted amino acid sequences from the random region of 113 affinity-selected bacteriophage were analyzed, and two dominant consensus motifs were identified (Table I). When compared with the amino acid sequence of p47phox, these motifs mapped to residues 319–332 and 361–370 in p47phox. The strongest homology among the phage peptides was evident in those representative of the p47phox region SDDAYRNSVRFL, which represents a putative novel site of interaction between p47phox and p67phox. As Table I shows, 19 phage isolates contained 3–4 residues identical to this region, and 8 other phage isolates supported this consensus. Additionally, the presence of conservative substitutions in these phage sequences compared with the p47phox sequence suggests an even greater similarity of many of the phage peptides to this region. Interestingly, our previous screening of purified flavocytochrome b, using a similar experimental approach, demonstrated that this same region in p47phox residues 323–342, represented a part of a flavocytochrome b binding domain (30).

The second consensus sequence mapped to p47phox 360–380 and was represented by 11 phage peptides (see Table I). Previously, Leto et al. (28) and Finan et al. (31) demonstrated that the proline-rich region encompassing p47phox residues 358–371 of p47phox was a site of interaction with p67phox and recently proposed that this region bound to the second SH3 domain of p67phox in resting neutrophils (34, 36). Thus, our data mapping this region of p47phox as a p67phox interactive site not only confirms the previous results of Leto et al. (28) and Finan et al. (31) but also serves as a positive control for our phage-display library analysis. Since this site has previously been characterized (28, 31, 34, 36), no further analyses were performed involving this region of p47phox.

Peptide Mapping and Affinity Binding Assays—Previously, we reported that p47phox residues 323–342 were important for association with flavocytochrome b, and peptides mimicking this region blocked interaction of p47phox with flavocytochrome b (30). However, based on the data presented above from the phage-display analysis of p67phox, this same region of p47phox also appears to be a binding site for p67phox, suggesting that following activation, p67phox and flavocytochrome b may compete for p47phox through a high affinity interaction involving p47phox residues 323–332. To evaluate this hypothesis, we analyzed synthetic peptides mimicking this region of p47phox for their ability to block p47phox-p67phox binding using affinity binding assays. As shown in Fig. 1A, little or no p67phox binds to immobilized p47phox prior to activation with SDS, but this binding significantly increased (up to 100-fold) after activation of the system with SDS. Similarly, p47phox bound to immobilized p67phox only after activation with SDS. As controls for nonspecific binding or aggregation of cytosolic proteins with the Sepharose beads, we performed similar experiments using unconjugated and BSA-.

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Table I

| Phage Peptide | Residues | Consensus Motif |
|---------------|----------|-----------------|
| 1             | 319–332  | SDDAYRNSVRFL    |
| 2             | 323–342  | 360–380         |
| 3             | 358–371  | 319–332 or 323–342 |

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Analysis of p47<sub>phox</sub>-p67<sub>phox</sub> Binding Interactions

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Preassembled complexes of p47Phpox with either p67Phpox or flavocytochrome b are not dissociated by p47Phpox peptide (25) and S-AYRRNSVRFL (32). Sepharose conjugated with p47Phpox or p67Phpox (S-p47 and S-p67, respectively) were incubated with human neutrophil cytosol (cytosol), recombinant p47Phpox (rp47), recombinant p67Phpox (rp67), or human neutrophil membranes (membranes), and 1 mM AYRRNSVRFL was added either before or 1.5 h after SDS activation as described under "Materials and Methods." Bound proteins were then determined by SDS-PAGE and Western blotting with the appropriate antibody as indicated. The results are displayed as a percent of control (stimulated assay with no peptide) and represent the mean ± S.D. of (n) experiments. ND, experiment not performed.

| Assay composition | Unstimulated assay | 1 mM AYRRNSVRFL added before SDS | 1 mM AYRRNSVRFL added 1.5 h after SDS | 1 mM DCTDLESTRREV added before SDS | % of control | % of control | % of control | % of control |
|-------------------|-------------------|---------------------------------|--------------------------------------|------------------------------------|-------------|-------------|-------------|-------------|
| S-p47 + cytosol (Blot α-p67Phpox) | 0.0 ± 0.0 (2) | 1.9 ± 1.4 (3) | 94.8 ± 13.1 (2) | 75.6 ± 5.8 (2) | 100.0 ± 10.3 (2) |
| S-p47 + rp67 (Blot α-p67Phpox) | 2.8 ± 3.9 (3) | 2.1 ± 2.0 (3) | 104.7 ± 23.9 (3) | 115.9 ± 19.6 (3) |
| S-p67 + cytosol (Blot α-p47Phpox) | 2.0 ± 0.0 (3) | 3.4 ± 2.4 (3) | 125.2 ± 30.7 (3) | 147.9 ± 7.4 (3) |
| S-p67 + rp47 (Blot α-p47Phpox) | 5.8 ± 3.1 (2) | 9.7 ± 3.9 (2) | 123.9 ± 25.9 (3) | 115.9 ± 19.6 (3) |
| S-p47 + membranes (Blot α-p22Phpox) | 7.2 ± 5.2 (3) | 18.9 ± 9.5 (2) | 138.2 ± 13.6 (3) | 228.8 ± 72.3 (3) |
| S-p47 + membranes (Blot α-gp91Phpox) | 1.6 ± 2.3 (3) | 8.5 ± 6.0 (3) | 104.7 ± 23.9 (3) | 115.9 ± 19.6 (3) |

conjugated Sepharose beads. The results shown in Fig. 1B demonstrate that there was essentially no nonspecific binding or precipitation of p47Phpox or p67Phpox from human cytosol to unconjugated or BSA-conjugated Sepharose beads under activating conditions (similar results were also observed using recombinant cytosolic proteins instead of cytosol (data not shown)).

Using the affinity binding assay described above, we analyzed the peptide identified by our phage-displayscreen. As shown in Fig. 1A, a peptide mimicking p47Phpox residues 232–AYRRNSVRFL inhibited binding of recombinant p47Phpox or p67Phpox to immobilized p67Phpox or p47Phpox, respectively, following activation (EC50 = 250 μM using either assay condition), whereas control peptides had no effect on this binding interaction. Additionally, this peptide blocked the association of p47Phpox or p67Phpox present in normal human neutrophil cytosol to the immobilized complimentary cytosolic protein when added at the time of activation (Fig. 1A). In contrast, addition of 1 mM peptide 323–AYRRNSVRFL to the preassembled p47Phpox-p67Phpox complex (100-fold molar excess with respect to p67Phpox) 1.5 h after activation failed to disrupt the complex (Table II), suggesting the formation of a high affinity interaction between p47Phpox and p67Phpox that cannot be disrupted by this peptide alone.

In our previous studies mapping a domain of p47Phpox that interacts with flavocytochrome b (30), we found that peptides representing regions flanking and/or partially overlapping the 323–332 site of p47Phpox also inhibited NADPH oxidase activity in cell-free assays and in electropherometric neutrophils. Therefore, because p47Phpox 323–332 blocked the association of p47Phpox and p67Phpox, we analyzed these peptides in our affinity binding assay system. As shown in Fig. 1A, we found the peptides flanking/partially overlapping our active region, 315SRKLSQDAYRNS232 and 339QRRQARQGPSQPG347, and p67Phpox carboxyl-terminal peptide 511DCTDLESTR_EV526 had no effect on the p47Phpox-p67Phpox association when tested up to 1 mM peptide (Fig. 1A, lanes 8 and 9, and Table II, respectively). The inability of these peptides, which partially overlap the active region (e.g. peptide 315–328 overlaps 60% of peptide 323–332) and/or have an even greater number of charged residues, to inhibit formation of the p47Phpox-p67Phpox complex provides further evidence for a sequence and/or conformational specificity in peptide 323–332 for interfering with the formation of the p47Phpox-p67Phpox complex.

As mentioned above, p47Phpox residues 323–332 have been shown previously to be important for association with flavocytochrome b using cell-free oxidase translocation assays (30, 45). Because our affinity binding assays indicated this site also interacts with p67Phpox, we tested the peptide for its ability to block p47Phpox-flavocytochrome b association in the same in vitro affinity binding assay to confirm that p47Phpox 323–332 was indeed blocking both interactions in the same assay system. As shown in Fig. 2, this peptide inhibited flavocytochrome b binding to p47Phpox at similar or slightly higher concentrations than those required to block the p47Phpox-p67Phpox association (IC50 values of ~400 and 200 μM, respectively), whereas two control peptides (slightly smaller and larger than 323–AYRRNSVRFL) had no inhibitory effect, indicating that the inhibition exhibited by peptide 323–332 is sequence-specific. The data shown in Fig. 2 suggest that flavocytochrome b binds to p47Phpox with a slightly higher affinity than does p67Phpox; this would be expected given the multi-site binding interaction that occurs between p47Phpox and both subunits of flavocytochrome b (24–29). Finally, addition of 1 mM peptide 323–332 to assays containing complexed p47Phpox and flavocytochrome b 1.5 h after activation failed to disrupt the p47Phpox-flavocytochrome b complex (Table III), confirming the formation of a stable complex that is resistant to peptide disruption (46).

**Competition Assays—** Based on our results, it appears that both flavocytochrome b and p67Phpox compete for binding to p47Phpox, possibly at residues 323–332. To evaluate this possibility, we analyzed the ability of flavocytochrome b to compete with p67Phpox for binding to p47Phpox using competition assays. As shown in Fig. 3, addition of p67Phpox to immobilized p47Phpox at an approximate 1:1 molar ratio prior to addition of flavocytochrome b (flavocytochrome b was added at 1.5 h after activation of the assay containing p47Phpox and p67Phpox) almost completely blocked (~91% inhibition) flavocytochrome b binding to p47Phpox compared with controls, supporting the formation of a stable complex that renders the 323–332 binding region inaccessible as indicated by our peptide studies.

Similarly, addition of flavocytochrome b to p47Phpox prior to addition of p67Phpox, even at a much lower molar ratio (~5 times less flavocytochrome b, assuming 3 × 106 flavocytochrome b molecules/cell), reduced p67Phpox binding to p47Phpox by ~80% (Fig. 3). The results confirm that flavocytochrome b and p67Phpox do indeed compete for binding to p47Phpox.

**Peptide Effects in the Cell-free NADPH Oxidase Assay—** In previous studies, Nauseef et al. (45) found that p47Phpox peptide 323–332 inhibited O2– in a cell-free NADPH reconstitution assay system and blocked assembly of the oxidase in a cell-free translocation assay. Recently, we confirmed this result and further demonstrated that this peptide directly inhibited the association of p47Phpox with flavocytochrome b (30). To further evaluate the site(s) of action of this peptide, we manipulated the cell-free assay following the method of Kleinberg et al. (46). As shown in Table III, p47Phpox peptide 323–332 inhibited NADPH oxidase activity in a cell-free assay containing all NADPH oxidase cytosolic components when added before or simultaneously with SDS activation (i.e. prior to NADPH oxidase assembly) but loses its ability to inhibit NADPH oxidase activity when added at >5 min after activation, indicating that...
the site of action of this peptide becomes inaccessible after assembly of the NADPH oxidase. As we observed previously, however, there is a variable lag time after activation where the peptide is partially inhibitory, presumably due to the presence of unassembled, partially assembled, and/or unstable oxidase complexes. At longer time points after activation, fully assembled, partially assembled, and/or unstable oxidase complexes are resistant to peptide effects. However, there is a variable lag time after activation, where the peptide is partially inhibitory, presumably due to the presence of unassembled, partially assembled, and/or unstable oxidase complexes. At longer time points after activation, fully assembled oxidase complexes are resistant to peptide effects.

To further investigate the site of action of p47phox peptide 323–332, peptide was added to assays deficient in either p47phox or p67phox following activation with SDS and simultaneously with the missing cytosolic factor, either p47phox or p67phox, at 5 and 10 min after SDS addition. As shown in Table III, addition of peptide concurrently with p67phox and membranes had no inhibitory effect on O2- production. In contrast, addition of peptide simultaneously with p47phox at 5 and 10 min after activation of a mixture containing only p67phox and membranes inhibited O2- production by –78.7 and 91.7%, respectively (Table III). This level of inhibition is similar or even better than that observed if the peptide is added before activation of the assay containing membranes and both p47phox and p67phox (Table III). Interestingly, when the activation mixture contained only p47phox and membranes, and p67phox (with or without peptide) was added after activation, O2- production was significantly higher than in control values (Table III). This phenomenon was previously observed by Kleinberg et al. (46) although it was not discussed in their report. In the context of our present studies, however, this result suggests that p67phox may be serving a regulatory function via its binding to p47phox 323–332.

**DISCUSSION**

The nature of the p47phox-p67phox interaction and its function in human neutrophils is not well understood. Activation in neutrophils and in vitro using a cell-free assay system appears to cause a conformational change or unfolding of an active domain(s) in p47phox, allowing it to associate with or form a higher affinity association with p67phox and then translocate to the membrane where both stably associate with the active NADPH complex. Several recent reports indicate that these p67phox-p47phox complexes may be mediated by SH3 domain interactions (27, 28, 31, 32, 34-36) as p47phox and p67phox
each have two SH3 domains and two proline-rich SH3 targets (14, 15, 27, 28, 31, 32, 47). It has recently been reported that the carboxyl-terminal regions of both proteins were necessary for the formation of a p67^phox-p47^phox complex (28, 31, 34, 36) in resting cells and that the amino-terminal SH3 domain of p47^phox interacts preferentially with p22^phox (35). However, the requirement for these interactions in initiating O_2 production is uncertain as deletion of both p67^phox SH3 domains has no effect on in vitro O_2 generation (32, 33), indicating that SH3-mediated interactions are only partially responsible for assembly of the oxidase components and activation of the NADPH oxidase.

In addition to SH3-mediated interactions, increasing evidence indicates there are other important functional sites of interaction between NADPH oxidase components, and these interactions are characterized by significantly higher affinities than those of the SH3 interactive domains (24, 25, 29, 30, 45). Phosphorylation also plays a role in activation of the NADPH oxidase in vivo (3, 48–51) and may initiate a conformational change within p47^phox. There are eight or nine p47^phox phosphorylation sites within residues 303–379, including Ser-320 and Ser-328 which are in close proximity to the site of interaction identified in this report (50, 52, 53), and it has been proposed that phosphorylation may serve to neutralize the p47^phox carboxy-terminal domain encompassing residues 314–347, thus allowing it to interact with the membrane or target protein.

In an effort to further understand the interaction between p47^phox and p67^phox, we have utilized several approaches to identify and characterize potential sites of interaction of these proteins. Our present studies demonstrate the presence of a novel site in p47^phox (residues 323–332) that binds p67^phox after activation of the NADPH oxidase. This site was mapped using random sequence phage-display library analysis of p67^phox and peptide mapping using protein affinity binding assays and in vitro NADPH oxidase assays. These experiments demonstrated that a peptide mimicking p47^phox residues 323–332 completely blocked p47^phox-p67^phox association, whereas peptides representing flanking regions had no inhibitory effect. Thus, in addition to the interaction between the second SH3 domain of p67^phox and the proline-rich carboxyl-tail of p47^phox, which is present in resting cells, a further interaction between these proteins at p47^phox residues 323–332 is induced during activation.

Previously, we found that p47^phox residues 323–342 represented a site of binding interaction with flavocytochrome b(30), and this observation was confirmed in the present report using two different in vitro assays that were not evaluated previously. Thus, taken together our data suggest that both p67^phox and flavocytochrome b utilize a common binding site in p47^phox, presumably at distinct stages of the activation process. Using affinity binding assays, we confirmed that p67^phox and flavocytochrome b do indeed compete for binding to p47^phox and further demonstrated that p47^phox 323–332 bound with high affinity to flavocytochrome b after activation and became inaccessible to peptide. Prior to assembly, however, this region of p47^phox appears to be associated with p67^phox and at some point in the activation process is transferred to flavocytochrome b.

Previously, Leto et al. (28) reported that a GST-fusion protein containing p47^phox residues 280–338 did not bind to recombinant p67^phox, and this region of p47^phox encompasses the region we have reported here to associate with p67^phox. One explanation for this disparity is that GST-fusion proteins of this region of p47^phox do not accurately reflect conformational characteristics of the native protein and, therefore, are not capable of mediating a binding interaction between these molecules. In addition, Leto et al. (28) tested their interactions using nonactivating or resting cell conditions. In contrast, we observed specific interactions mediated by p47^phox 323–332 only after SDS activation, and none of the activation-induced conformational changes and/or charge neutralization in p47^phox and p67^phox would have been present in the assays of Leto et al. (28). Thus, studies using only nonactivated binding assays (28, 34) or yeast two-hybrid assays (19, 31, 36) seem to provide accurate information about binding interactions occurring between the NADPH oxidase proteins in the resting cell but may fail to detect activation-induced interactive sites. In support of this conclusion, de Mendez et al. (34) recently reported that the interaction between the second SH3 domain of p67^phox and p47^phox residues 358–171 occurs in the resting cell and, using phage-display library analysis, we confirmed their results (see Table 1). However, we also report the presence of a novel, higher affinity binding site for p67^phox in p47^phox that is only utilized under activating conditions.

The role of the p47^phox-p67^phox association in regulating NADPH oxidase assembly and activation is currently unknown. Using the cell-free NADPH oxidase assay, de Mendez et al. (32) and Leusen et al. (33) found that the interaction between the carboxyl-terminal proline-rich region of p47^phox and the second SH3 domain of p67^phox was not required for activity in vitro but that this interaction was necessary for in vivo oxidase activity (32). Similarly, our peptide analysis in the cell-free NADPH oxidase assay suggests that the association of p47^phox with p67^phox via p47^phox residues 323–332 is also not

### Table I

| Assay composition | 5-min SDS incubation | 10-min SDS incubation |
|-------------------|----------------------|----------------------|
| Membranes + p47^phox (p67^phox-deficient) | 0.18 ± 0.23 (7) | 0.06 ± 0.18 (9) |
| Membranes + p67^phox (p47^phox-deficient) | 0.39 ± 0.47 (7) | 0.24 ± 0.36 (9) |
| Membranes + p47^phox + p67^phox (control peptide added before SDS) | 101.6 ± 10.4 (2) | 96.8 ± 0.6 (2) |
| Membranes + p47^phox + p67^phox (control peptide added after SDS) | 102.6 ± 2.7 (2) | 113.9 ± 5.5 (2) |
| Membranes + p47^phox + p67^phox (Ayrnysrfl332 added after SDS) | 35.1 ± 10.3 (4) | 34.1 ± 1.7 (4) |
| Membranes + p47^phox + p67^phox (Ayrnysrfl332 added after SDS) | 73.5 ± 1.9 (4) | 99.3 ± 6.5 (4) |
| Membranes + p67^phox (p47^phox added after SDS) | 69.6 ± 8.4 (2) | 76.0 ± 3.7 (6) |
| Membranes + p67^phox (p47^phox added before SDS) | 21.3 ± 3.0 (7) | 8.3 ± 3.4 (6) |
| Membranes + p47^phox (p67^phox added after SDS) | 110.8 ± 6.5 (6) | 108.1 ± 13.6 (6) |
| Membranes + p47^phox (p67^phox + Ayrnysrfl332 added after SDS) | 119.1 ± 6.5 (5) | 128.0 ± 15.6 (6) |

*aThe sequence of the control peptide was AVEGGMKPVKLLVGC. The results are expressed as percent of control O_2-generating activity (158–210 nmol of O_2/min/mg of membrane protein for a complete assay system with no added peptides) and represent the mean ± S.D. of (n) experiments.*
Analysis of p47<sub>phox</sub>-p67<sub>phox</sub> Binding Interactions

which would bind to p67<sub>phox</sub> (represented by box B in Fig. 4) and be sequestered from nonspecific interactions during translocation to the site of NADPH oxidase assembly. This translocation process seems to be mediated both by increased p47<sub>phox</sub> phosphorylation (49, 50) and low affinity SH3 interactions between p47<sub>phox</sub> and other NADPH oxidase components (28, 31, 35, 36) (represented by box C in Fig. 4). At the site of assembly, p67<sub>phox</sub> would then be switched off of this active site, possibly by the action of small GTP-binding proteins (Rac or Rap1A) or some other stimulus, exposing it for gp91<sub>phox</sub> binding (represented by box D in Fig. 4) at a larger domain of p47<sub>phox</sub> encompassing residues 323–342 and overlapping the previously occupied p67<sub>phox</sub> binding site. Complementary binding sites in gp91<sub>phox</sub> encompassing residues 85–93, 450–451, 491–504, and 559–565 presumably mediate this interaction with p47<sub>phox</sub> (25, 29, 46).

In addition to escorting p47<sub>phox</sub> p67<sub>phox</sub> must play some other functional role in the NADPH oxidase, as p47<sub>phox</sub>, Rac, and flavocytochrome b alone cannot support O<sub>2</sub> production in vivo and require the addition of p67<sub>phox</sub> even after translocation and assembly of the other essential NADPH oxidase components (in which case p47<sub>phox</sub> residues 323–332 would be inaccessible for p67<sub>phox</sub> binding). Such a distinct regulatory role for p67<sub>phox</sub> is supported by the significantly higher levels of O<sub>2</sub> observed in cell-free NADPH oxidase assays where p67<sub>phox</sub> was added after assembly of p47<sub>phox</sub> with flavocytochrome b. Additionally, Tamura et al. (54) found that chemically cross-linked NADPH oxidase complex was stabilized by p47<sub>phox</sub> but were unstable if they contained p67<sub>phox</sub> in the absence of p47<sub>phox</sub>. Finally, Cross and Curnutte (55) recently reported that p47<sub>phox</sub> and p67<sub>phox</sub> played distinct roles in controlling electron flow from NADPH to oxygen, and our present report supports their observations and provides further clues to explain how these proteins carry out their distinct functional roles.

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