A Domain of p47phox That Interacts with Human Neutrophil Flavocytochrome b<sub>55b</sub>*

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The NADPH-dependent oxidase of human neutrophils is a multicomponent system including cytosolic and membrane proteins. Activation requires translocation of cytosolic proteins p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac2 to the plasma membrane and association with the membrane flavocytochrome b to assemble a functioning oxidase. We report the location of a region in p47<sup>phox</sup> that mediates its interaction with flavocytochrome b. From a random peptide phage display library, we used biopanning with purified flavocytochrome b to select phage peptides that mimicked potential p47<sup>phox</sup> binding residues. Using this approach, we identified a region of p47<sup>phox</sup> from residue 323 to 342 as a site of interaction with flavocytochrome b. Synthetic peptides 323AYRNRNSVRFL332, 323AYRNRNSVRFL332, and 323AYRNRNSVRFL332 inhibited superoxide (O<sub>2</sub>) production in the broken cell system with IC<sub>50</sub> of 18, 57, and 15 μM, respectively. 323AYRNRNSVRFL332 and its derivative peptides inhibited phosphorylation of p47<sup>phox</sup>. However, the functional importance of this peptide was independent of its effects on phosphorylation, since 323AYRNRNSVRFL332 inhibited O<sub>2</sub> production, but had no effect on phosphorylation. None of the peptides blocked O<sub>2</sub> production when added after enzyme activation, suggesting that they inhibited the assembly, rather than the activity, of the oxidase. Furthermore these peptides inhibited membrane association of p47<sup>phox</sup> in the broken cell translocation assay and O<sub>2</sub> production by electropor-膜ilized neutrophils, thereby supporting the interpretation that this region of p47<sup>phox</sup> interacts with flavocytochrome b.

Human polymorphonuclear leukocytes (PMNs)<sup>4</sup> play an important role in host defense against invading microorganisms. PMNs possess an NADPH-dependent oxidase which is capable of generating superoxide anion (O<sub>2</sub>) and other microbial oxygen-derived species (e.g., H<sub>2</sub>O<sub>2</sub>, HOCl) when activated by various particulate and soluble stimuli (1, 2). The NADPH oxidase is a multicomponent enzyme system which is assembled in resting PMNs but assembles on the plasma membrane in activated PMNs (3, 4). The critical importance of the PMN NADPH oxidase in normal host defense is most dramatically illustrated by the frequent and severe infections seen in patients with chronic granulomatous disease (5, 6). The PMNs from such patients lack a functionally competent oxidase and, when stimulated, fail to generate O<sub>2</sub>.

Essential components of the NADPH oxidase include plasma membrane and cytosolic proteins. The key plasma membrane component is a heterodimeric flavocytochrome b which is composed of a 91-kDa glycoprotein (gp91<sup>phox</sup>) and a 22-kDa protein (p22<sup>phox</sup>) (7–10). Flavocytochrome b serves to transfer electrons from NADPH to molecular oxygen, resulting in the generation of O<sub>2</sub>. In PMN membranes, a low molecular weight GDP-binding protein, Rap1A, is associated with flavocytochrome b and plays an important role in NADPH oxidase regulation in vivo (11–13). Cytosolic proteins p47<sup>phox</sup>, p67<sup>phox</sup>, and a second low molecular weight GDP-binding protein, Rac2, are absolutely required for NADPH oxidase activity (14–18), and these three proteins associate with flavocytochrome b to form the functional NADPH oxidase (19–21). Additionally, a cytosolic protein, p40<sup>phox</sup>, has recently been identified, but its role in oxidase function is not completely defined (22).

According to the current model of NADPH oxidase assembly, p47<sup>phox</sup> and p67<sup>phox</sup> translocate en bloc to associate with flavocytochrome b during PMN activation (23, 24). Rac2 translocates simultaneously but independently of the other two cytosolic components to associate with the membrane-bound NADPH oxidase (25, 26). Studies of oxidase assembly in PMNs of patients with various forms of chronic granulomatous disease suggest that p47<sup>phox</sup> binds directly to flavocytochrome b (20), and at least six regions of flavocytochrome b have been identified as potential sites for interaction with p47<sup>phox</sup>, including four sites on gp91<sup>phox</sup> and two sites on p22<sup>phox</sup> (27–34). In contrast, the complementary sites of interaction presented on p47<sup>phox</sup> have not been fully characterized (29, 33, 34). In previous studies, peptides mimicking p47<sup>phox</sup> residues 323AYRNRNSVRFL332 inhibited phosphorylation of p47<sup>phox</sup>, O<sub>2</sub> production, and translocation of cytosolic components in the broken cell system (35), suggesting that this might be a possible site of interaction between p47<sup>phox</sup> and flavocytochrome b.

In the present work, we used an approach combining the screening of a random peptide phage display library with the functional analysis of synthetic peptides to define residues in p47<sup>phox</sup> which interact with flavocytochrome b. Our data indicate that the region encompassing amino acids 323–342 comprises a functionally important domain in the association of p47<sup>phox</sup> with flavocytochrome b.
**Domain of p47\textsuperscript{phox} That Interacts with Flavocytochrome b**

**EXPERIMENTAL PROCEDURES**

Materials—NADPH, ferricytochrome c (horse heart, type V1), superoxide dismutase, and FAD were obtained from Sigma. GTP-S (5'-triphosphate) was obtained from Boehringer Mannheim and arachidonic acid (sodium salt) from NuCheek Prep (Elyssian, MN). NHS-LC-biotin was obtained from Pierce, and streptavidin was from Fisher. Dextran, Ficoll, and Percoll were obtained from Pharmacia Biotech Inc., and Hypaque was obtained from Winthrop Laboratories (New York).

Preparation and Fractionation of PMNs—Human PMNs were isolated from heparinized venous blood using sequential dextran sedimentation, differential density sedimentation in Hypaque-Ficoll gradients, and hypotonic lysis of erythrocytes as described previously (35, 36). Two different methods were employed for the isolation of PMN membranes and cytosol with no difference in the experimental results. Purified PMNs were treated with 2% deoxyribofuranosyl fluoride for 20 min at 4°C, washed, resuspended in relaxation buffer (KCl, 100 mM; NaCl, 3 mM; MgCl\(_2\), 3.5 mM; EGTA, 1.25 mM; Pipes, 10 mM, pH 7.3) and disrupted by N\(_2\) cavitation (35). Nuclei and unbroken cells were pelleted (200 x g, 6 min, 5°C) and the supernatant loaded on top of an isotonic discontinuous gradient of Percoll as described previously (37). Cytosol was collected from the top of the gradient and clarified before use. The plasma membrane fraction was collected, Percoll removed by centrifugation, and the membrane washed prior to use. Alternatively, purified PMNs (5 x 10\(^6\)/ml in 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 15 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml chymostatin, 1 mM EGTA, and 10 mM Heps, pH 7.0) were disrupted by N\(_2\) cavitation as described previously. Membrane and cytosolic fractions were prepared from the cytosate by sequential centrifugation as described by Fujita et al. (36).

Preparation and Purification of Flavocytochrome b—Human neutrophil flavocytochrome b was purified from ~10\(^{10}\) neutrophils following the methods of Parkos et al. (7, 38). The purified flavocytochrome b was >95% pure, as determined by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (7, 38), and immunoblotting with anti-cytocrome b antibodies confirmed the presence of both gp91\textsuperscript{phox} and p22\textsuperscript{phox} (not shown).

Random Sequence Peptide Bacteriophage Display Library Analysis—Fifty \(\mu\)g of human neutrophil flavocytochrome b was biotinylated to a final biotin:protein ratio of 20:1 (39). Three rounds of biopanning were performed as described previously (39) using 10 \(\mu\)g/ml of biotinylated flavocytochrome b in 0.2% Triton X-100 and 75 \(\mu\)l (round 1 only) of a nonapeptide phage display library (404-3) (40). The bacteriophage eluates of the first two pans were amplified in K9 Escherichia coli cells on solid Luria-Bertani (LB) agar dishes containing 100 \(\mu\)g/ml of biotinylated flavocytochrome b as the activating agent, and activity was determined as described by Nauseef et al. (35). In other experiments, activity was measured in an SDS-polyacrylamide gel electrophoresis and electroblotted as described previously (21, 35). Immunoblots were probed with primary antibody, rabbit anti-p47\textsuperscript{phox} (21, 35, 44), and followed by either iodinated protein A or alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad). Relative amounts of translocated proteins were quantitated by densitometric analysis of the immunoreactive p47\textsuperscript{phox} detected by autoradiography or colorimetric assay.

Preparation and E. coli permeabilization of Human Neutrophils—Histopaque-purified neutrophils were washed once with ice-cold permeabilization buffer (140 mM KCl, 10 mM Heps, 10 mM glyc-MgCl\(_2\), 0.193 mM CaCl\(_2\), and 1 mM EGTA, pH 7.2) and then resuspended in the same buffer supplemented with 100 \(\mu\)M GTP•S, 1 mM ATP, and 2 mM NADPH, to a final concentration of 10\(^7\) cells/ml and stored on ice. Aliquots of 800 \(\mu\)l were placed in 0.4-cm electroporation cuvettes and permeabilized with a Bio-Rad gene pulse using two consecutive pulses (with brief stirring between pulses) of 3.75 kV/cm with a 25-microfarad capacitor (\(\approx\) 0.3–0.5 ms) at 4°C. The cells were then incubated on ice for 5 and 30 min with or without 1 mM of the appropriate peptide. Subsequently, the cells were resuspended into permeabilization buffer containing 100 \(\mu\)M cytochrome c (+1 mM peptide) (final concentration of 5 x 10\(^4\) cells/ml) and then stimulated with 1 \(\mu\)M horseradish peroxidase 12-myristate 13-acetate (reference cuvette contained 25 \(\mu\)g/ml superoxide dismutase). The maximal rate of superoxide dismutase-inhibitable reduction of cytochrome c was determined on a Cary dual-beam spectrophotometer (Varian, Melbourne, Australia) at 550 nm and 25°C for 10 min. Electroporated neutrophils retained 92.3 ± 1.9 and 49.8 ± 6.3% of their ability to produce O\(_2\) when incubated for 5 and 30 min after pulsing, respectively, compared with control, nonpermeabilized cells and were found to be 98% permeable to trypan blue 5 min after permeabilization.

**RESULTS**

Biopanning with Purified Flavocytochrome b—to identify the region or regions in p47\textsuperscript{phox} that associate with flavocytochrome b, a random sequence nonapeptide bacteriophage display library was screened with purified flavocytochrome b. The predicted amino acid sequences from 94 of these affinity selected bacteriophage were analyzed and three dominant consensus motifs were identified (Fig. 1). When compared with the amino acid sequence of p47\textsuperscript{phox}, these motifs mapped to residues 323–342 in p47\textsuperscript{phox}, a region which contains multiple sites for serine/threonine kinase phosphorylation and includes a previously identified area of potential interaction with flavocytochrome c. At residues 323–332 (35, 45), the strongest homology among phage peptides was evident in those representative of the p47\textsuperscript{phox} region (338) and eight phage isolates contained three to four residues identical to this region. Additionally, seven phage isolates contained peptides that were homologous to p47\textsuperscript{phox} residues 333–342 (38) (six of these contained three-residue matches), which immediately precede the 338–342 region. In combination, 21 phage isolates were representative of the p47\textsuperscript{phox} region 338–342 (Fig. 1). Conservative substitutions and one residue shifts give many phage representative of the p47\textsuperscript{phox} region 335–337, where arginine appears to be substituted by a positively charged histidine or lysine.

A third consensus sequence mapped to p47\textsuperscript{phox} residues 324–328 and was represented by 21 phage peptides, and conservative substitutions or one-residue shifts give many phage representative of this area even greater similarity. Nauseef et al. (35) previously found that p47\textsuperscript{phox} residues 324–328 represented a functionally important domain involved in NADPH oxidase assembly. Certain residues within this region appear to be critically important for structural constraints, as the exclusion of these residues renders the peptide ineffective for various peptides on the phosphorylation of p47\textsuperscript{phox} mirrored closely those for quantification of \(\text{O}_2\) generation and have been described in detail previously (35). Relative quantitation of p47\textsuperscript{phox} phosphorylation was obtained by densitometric analysis of resultant autoradiographs.

Electrophoresis and Immunoblotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis and electroblotted as described previously (21, 35). Immunoblots were probed with primary antibody, rabbit anti-p47\textsuperscript{phox} (21, 35, 44), and followed by either iodinated protein A or alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad). Relative amounts of translocated proteins were quantitated by densitometric analysis of the immunoreactive p47\textsuperscript{phox} detected by autoradiography or colorimetric assay.
Production in the broken cell assay (35). Thus, production in the broken cell system. Charge productions significantly in the broken cell system (Table I). Peptide phages were sequenced and analyzed in a previous study. Representative of these regions were synthesized and analyzed in the broken cell assay at concentrations up to 500 μM. ![Peptide Inhibition in the Broken Cell Assay—](Image)

**Domain of p47phox That Interacts with Flavocytochrome b**

**Phage Sequences**

| Phage Sequence | X3 |
|----------------|----|
| LTVAQVRPG     |    |
| APVAPFVV      |    |
| SVGWEEEH      |    |
| X3 |
| X3 |
| HENQEGQA      |    |
| TQVEQAL       |    |
| X3 |
| X3 |
| SRRGSTQS      |    |
| NNQGQPNS      |    |
| X3 |
| X3 |
| FNLLGWSLEQ   |    |
| VQANWGRF      |    |
| X3 |
| X3 |
| LGNTRPSV      |    |
| LYSIQHDF      |    |
| X2 |
| X2 |
| AAEKQHQH      |    |
| ANQYVQRG      |    |
| TRKVEQG      |    |
| VLSQVWNO      |    |
| FVQSGASG     |    |
| HYETGLGR      |    |
| SGRKVRPL      |    |
| X3 |
| X3 |
| SAVGRKVR      |    |
| AGQGRKQR      |    |
| NUTPRENY      |    |
| X2 |
| X2 |
| TMQHECHS      |    |
| RMNRRIGF      |    |
| HNRSPDNL      |    |
| HEHSSNOV      |    |
| SNGHSNORM     |    |
| TSQDLFTK      |    |

**FIG. 1. Flavocytochrome b-binding phage displaying sequences of homology with p47phox.** The random regions of bacteriophage recovered from flavocytochrome b biopanning were sequenced and the putative motifs aligned. Residues identical to the corresponding p47phox sequence are underlined, conservative substitutions are in bold, and residues shifted in position are represented by italics. Bacteriophage peptide sequences representative of p47phox regions indicated are present in both NH₃ → COOH and COOH → NH₃ forms, and the X2/X3 designations indicate the number of clones recovered with that specific sequence.

At inhibiting O₂ production in the broken cell assay (35). Thus, the mapping of this site using a random sequence library provides direct evidence confirming the biological relevance of this site.

**Peptide Inhibition in the Broken Cell Assay—**To determine if the p47phox regions selected from the phage display library approach were relevant for the association of p47phox with flavocytochrome b and, thus, NADPH oxidase activity, peptides representative of these regions were synthesized and analyzed in a broken cell NADPH oxidase assay. In a previous study, p47phox peptides 299PPRRSSIRNA308 and 312HQRSRSRKRLSQD322 did not affect O₂ production significantly in the broken cell assay at concentrations up to 500 μM (35). These peptides encompass potential phosphorylation sites, are also positively charged, and precede the 323–342 region of p47phox mapped by the phage display library. In contrast, the peptides mimicking portions of p47phox residues 315–347, 315SRKRLSQDAYRRNS328, and 334QRRQARPGPQSPG347, all inhibited O₂ production in the broken cell assay in a dose-dependent manner with IC₅₀ values of 18, 57, and 15 μM, respectively (see Fig. 2 and Table I). Surprisingly, a shorter peptide derived from 334QRRQARPGPQSPG347, 337QARPGP344, was much less inhibitory (IC₅₀ = 750 μM) (see Fig. 2). Apparently, the loss of key flanking residues and/or the potential phosphorylation site at Ser345, or most likely, loss of conformational constraint rendered 337QARPGP344 ineffective at inhibiting O₂ production in the broken cell system. Charge alone does not seem to be the major determinant of inhibition in the broken cell assay. A control, unrelated peptide (KLSPRDSNE) and two p47phox peptides (299PPRRSSIRNA308 and 315SRKRLSQDAYRRNS328) did not inhibit O₂ production significantly in the broken cell system at concentrations up to 500 μM (35) (see Fig. 2 and Table I).

### TABLE I. Analysis of p47phox-related peptides in the broken cell system

| Peptide       | IC₅₀ (μM) | Control phosphorylation (%) | Control, translocation (%) |
|---------------|----------|------------------------------|----------------------------|
| AYRRNVSFL     | 57 (3)   | 21.0 ± 0.8 (24)             | 16.0 ± 1.8 (6)             |
| YRRNVSVR      | >400 (4) | >23.1 ± 1.3 (4)             | 135.0 ± 2.5 (3)            |
| YRRNVSVF      | >500 (4) | >12.2 ± 1.1 (3)             | 104.0 ± 1.3 (3)            |
| YRRNVSVR      | 145 ± 6 (3) | >22.5 ± 2.2 (6)         | 39.0 ± 1.1 (3)             |
| AYRRNVSFL     | 33 ± 17 (3)  | >11.3 ± 1.0 (9)           | 17.0 ± 1.0 (3)             |
| AYRRNVSFL     | 45 ± 4 (3)   | >26.4 ± 2.0 (9)           | 14.3 ± 4.9 (3)             |
| AYRRNVSFL     | 76 ± 22 (4)  | >116.0 ± 2.1 (9)          | 19.0 ± 0.6 (3)             |

| Peptide       | IC₅₀ (μM) | Control phosphorylation (%) | Control, translocation (%) |
|---------------|----------|------------------------------|----------------------------|
| AYRRNVSFL     | 1.1 (3)  | 104.0 ± 4.5 (24)            | 16.0 ± 1.8 (6)             |
| YRRNVSVR      | >150 (4) | >23.1 ± 1.3 (4)             | 135.0 ± 2.5 (3)            |
| YRRNVSVF      | >500 (4) | >12.2 ± 1.1 (3)             | 104.0 ± 1.3 (3)            |
| YRRNVSVR      | 145 ± 6 (3) | >22.5 ± 2.2 (6)         | 39.0 ± 1.1 (3)             |
| AYRRNVSFL     | 33 ± 17 (3)  | >11.3 ± 1.0 (9)           | 17.0 ± 1.0 (3)             |
| AYRRNVSFL     | 45 ± 4 (3)   | >26.4 ± 2.0 (9)           | 14.3 ± 4.9 (3)             |
| AYRRNVSFL     | 76 ± 22 (4)  | >116.0 ± 2.1 (9)          | 19.0 ± 0.6 (3)             |

**Fig. 2. Effect of phage-mapped p47phox peptides on NADPH oxidase activity in the broken cell system.** Peptides 334QRRQARPGPQSPG347, 315SRKRLSQDAYRRNS328, and a control peptide KLSYRPDRSNE were added to the broken cell NADPH oxidase assay system at the indicated concentrations, and O₂ generation was measured as described under "Experimental Procedures." The results are expressed as a percent of control activity and represent the mean ± S.D. of three separate experiments.
Substitution of tryptophan either for Phe\textsuperscript{311} or for Tyr\textsuperscript{324} in p47\textsuperscript{phox} peptide 323–332 did not alter the inhibitory effects of the parent peptide on \( \mathrm{O}_2^\cdot \) production. The substitution S328A, which did not block \( \mathrm{O}_2^\cdot \) production or inhibit phosphorylation but did block \( \mathrm{O}_2^\cdot \) assembly, suggested that these peptides blocked assembly of the NADPH oxidase rather than activity, these peptides were tested in the broken cell translocation assay at 100 \( \mu \)M. Both peptides inhibited the translocation of p47\textsuperscript{phox} to the membrane compared to a control sample (Fig. 3). Peptide 330QRRQARPQGSPQSPG\textsuperscript{347} inhibited translocation to \( \approx 4\% \) of control (Fig. 3), whereas peptides 315SRKRLSODAYRRNS\textsuperscript{332} and 325RRNSVR330 inhibited p47\textsuperscript{phox} translocation to the membrane to 35 and 16\% (Fig. 3 and Table I) of control, respectively. In contrast, peptides representative of p47\textsuperscript{phox} residues preceding and partially encompassing 315–342 (325RPRRSSIRENA\textsuperscript{308} and 323HQRSRSLQSD\textsuperscript{322}) did not inhibit \( \mathrm{O}_2^\cdot \) production or translocation of p47\textsuperscript{phox} to the membrane in broken cell assay systems (35).

Inhibition of Superoxide Production in Electropereabilized Neutrophils—The broken cell assay for \( \mathrm{O}_2^\cdot \) production does not mimic entirely, the NADPH oxidase activity in the intact PMN (45). For that reason, the ability of active p47\textsuperscript{phox} peptides to inhibit in electropereabilized neutrophils was evaluated. Peptides 315SRKRLSODAYRRNS\textsuperscript{328}, 325AYRRNSVR330, and 334QRRQARPQGSPQSPG\textsuperscript{347} were incubated with the permeabilized neutrophils for 5 min and phosphol 12-myristate 13-acetate-stimulated \( \mathrm{O}_2^\cdot \) production by these cells was determined. We chose a 5-min treatment time in these experiments for two reasons: 1) the cells were determined to be fully permeabilized neutrophils (45). For that reason, the ability of active p47\textsuperscript{phox} peptides to inhibit in electropereabilized neutrophils was evaluated. Peptides 315SRKRLSODAYRRNS\textsuperscript{328}, 325AYRRNSVR330, and 334QRRQARPQGSPQSPG\textsuperscript{347} were incubated with the permeabilized neutrophils for 5 min and phosphol 12-myristate 13-acetate-stimulated \( \mathrm{O}_2^\cdot \) production by these cells was determined. We chose a 5-min treatment time in these experiments for two reasons: 1) the cells were determined to be fully permeable by this time (>98\% permeable), and 2) at longer incubation times the permeabilized cells failed to retain a level of \( \mathrm{O}_2^\cdot \) generating capacity that reasonably reflected that in the intact cell (>92\% of control activity was maintained after 5 min of incubation, whereas <50\% activity was present after 30 min).

As shown in Table II, the peptides that were inhibitory in the broken cell assay system also inhibited \( \mathrm{O}_2^\cdot \) production in permeabilized neutrophils (40–50\% inhibition compared with controls). Similarly, peptides that were inactive in the broken cell assay (325RRNCSQARPPQG\textsuperscript{344} and 325RRNSVR\textsuperscript{330}) also had no effect on permeabilized cells and served as negative controls (Table II). In addition, we also tested the gp91\textsuperscript{phox} carboxyterminal peptide RGVHIF as a positive control, since it was shown previously by Kleinberg et al. (27) to inhibit oxidase activity in electropereabilized cells. Our data (Table II) were consistent with their results. Two irrelevant peptides (AVEG-GMVPVKLLVGC and KLSYRPDRSNE) were used to deter-
mine non spécifique peptide effects in permeabilized cells, and their molecular weights (1500.3 and 1364.5, respectively) were similar to that of 32AYRRNSVRFL32 (1281.6), 315SRKRLSDAYRRNS328 (1719.1), and 334QRRQARPQPSQPG347 (1590.8). Thus, in support of our findings in the broken cell assays, our data indicate that the active p47phox peptides identified represent sites which participate in the assembly of the active NADPH oxidase in vivo.

**DISCUSSION**

The cytosolic NADPH oxidase protein p47phox has been shown to associate with flavocytochrome b, and several sites have been identified in carboxy-terminal domains of both gp91phox and p22phox that are important for this interaction (27–31). Additionally, our recent studies suggest that p47phox also binds to a region close to the amino terminus of gp91phox (32). Recent studies have examined which regions in p47phox participate in the binding to flavocytochrome b. Two reports demonstrated that Src homology 3 domains in p47phox may interact with p22phox (29, 33, 34). In addition, Nauseef et al. (35) screened p47phox peptides containing phosphorylation sites and found that one of these peptides (323–332) represents a functionally important domain in p47phox. This peptide, 323AYRRNS332, inhibits O2 production, phosphorylation, and translocation of p47phox in the broken cell system (35). Thus, the data suggested that this p47phox domain might play a role in oxidase assembly, although no direct evidence supported this possibility.

In the present studies using random peptide phage display library analysis, we provide direct evidence that p47phox residues 323–332, as well as the adjacent region extending to residue 342, are important in the binding of p47phox to flavocytochrome b. Thus, the entire binding domain encompasses residues 323–342 of p47phox, and synthetic peptides representing these adjacent sites and overlapping with the 323–332 region were potent inhibitors of NADPH oxidase activity.

The phage display library analysis of potential flavocytochrome b-binding domains in p47phox indicated that among the 94 phage sequenced the strongest homologies to p47phox were 32AYRRNSVRFL32 (1281.6), and 32QRRQARPQPSQPG347. Moreover, p47phox peptides encompassing these residues (32AYRRNSVRFL32, 32SRKRLSDAYRRNS328, 323AYRRNSVRFL332, and 334QRRQARPQPSQPG347) all inhibited superoxide production and translocation of p47phox in the cell-free system. The inhibition of O2 production was shown to be dose-dependent and targetted assemblage of the oxidase rather than activity, as demonstrated by the inhibition of translocation by these peptides and their ineffectiveness when added after assembly of the oxidase.

In addition to inhibiting O2 production and translocation in the broken cell system, the p47phox peptides 315SRKRLSDAYRRNS328, 323AYRRNSVRFL332, and 334QRRQARPQPSQPG347 all inhibited O2 production significantly in intact, permeabilized neutrophils, suggesting that they do indeed represent biologically relevant sites involved in NADPH oxidase assembly. Electropermeabilized neutrophils, which retained nearly all of their ability to generate O2 when compared with nonpermeabilized cells, may provide a more accurate representation of the in vivo situation. The inhibitory effect of the p47phox peptides on O2 production in permeabilized neutrophils supports the data from the phage library analysis and broken cell system and confirms that p47phox residues 323–342 are important for oxidase activation.

Phosphorylation of p47phox is required for activation of the NADPH oxidase in neutrophils (46–48); however, this requirement for phosphorylation is not observed in the broken cell system (35, 45). Previously, it has been suggested that phosphorylation of p47phox may function, in part, to neutralize positively charged regions of the protein, thus allowing it to interact with the membrane or target protein. In the broken cell system, the addition of anionic detergent (SDS) or arachidonic acid appears to bypass the need for p47phox phosphorylation by imparting negative charge to the protein. Consistent with this hypothesis, the p47phox region identified here as an important domain for association with flavocytochrome b and NADPH oxidase assembly (323–342), is within a larger region (314–347) that contains 11 positively charged residues, one protein kinase C phosphorylation site (Ser328), and is surrounded by several other potential sites for phosphorylation by protein kinase C and tyrosine kinase (49). Previously, Joseph et al. (50) reported that any polybasic peptide (≥5 basic residues) could nonspecifically inhibit NADPH oxidase activity; however, they also found that an 11 residue peptide containing six lysines had no effect on oxidase activity. We have analyzed a number of polybasic peptides, including some peptides with the same number of basic residues as in our active peptides, that had no inhibitory effect on NADPH oxidase activity in a broken cell assay system (Ref. 35 and Table I) and in electroporemeabilized neutrophils (Table II). Thus, the inhibition of NADPH oxidase activity by the active p47phox peptides is specific to their sequences and/or the charge distribution represented by their sequences, and the apparent “nonspecific” inhibition of oxidase activity by polybasic peptides unrelated to the NADPH oxidase, as reported by Joseph et al. (50), is due to the blocking of specific binding interactions (in this case between p47phox and gp91phox) that involve basic amino acid-enriched domains on one or both of the interacting proteins.

In conclusion, the data presented, based on the complementary approaches of random peptide phage display library analysis and peptide inhibition in both broken cell and permeabilized cell systems, demonstrate that p47phox residues 323–342 interact with flavocytochrome b and are required for the association of these two NADPH oxidase components. The association is sequence-specific and appears to require certain conformational constraints. The elucidation of the sites of interaction between human neutrophil NADPH oxidase component proteins will lead to a further understanding of the regulation of this system.

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