Mapping of Functional Domains in p47<sub>phox</sub> Involved in the Activation of NADPH Oxidase by “Peptide Walking”

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The superoxide generating NADPH oxidase of phagocytes consists, in resting cells, of a membrane-associated electron transporting flavocytochrome (cytochrome b<sub>559</sub>) and four cytosolic proteins as follows: p47<sub>phox</sub>, p67<sub>phox</sub>, p40<sub>phox</sub>, and the small GTPase, Rac (1 or 2). Activation of the oxidase is consequent to the assembly of a membranelocalized multimolecular complex consisting of cytochrome b<sub>559</sub> and the cytosolic components. We used “peptide walking” (Joseph, G., and Pick, E. (1995) J. Biol. Chem. 270, 29079−29082) for mapping domains in the amino acid sequence of p47<sub>phox</sub> participating in the molecular events leading to the activation of NADPH oxidase. Ninety-five overlapping pentadecapeptides, with a four-residue offset between neighboring peptides, spanning the complete p47<sub>phox</sub> sequence, were tested for the ability to inhibit NADPH oxidase activation in a cell-free system. This consisted of solubilized macrophage membranes, recombinant p47<sub>phox</sub>, p67<sub>phox</sub>, and Rac, and lithium dodecyl sulfate, as the activator. Eight functional domains were identified and labeled a−h. These were (N- and C-terminal residue numbers are given for each domain) as follows: a (21−35); b (105−119); c (149−159); d (193−207); e (253−267); f (305−319); g (325−339), and h (373−387). Four of these domains (c, d, e, and g) correspond to or form parts of regions shown before to participate in NADPH oxidase assembly. Thus, domain c corresponds to a region on the N-terminal boundary of the first src homology 3 (SH3) domain, whereas domains d and e represent more precisely defined sites within the full-length first and second SH3 domains, respectively. Domain g overlaps an extensively investigated arginine-rich region. Domains a and b, in the N-terminal half of p47<sub>phox</sub>, and domains f and h, in the C-terminal half, represent newly identified entities, for which there is no earlier experimental evidence of involvement in NADPH oxidase activation. “Peptide walking” was also applied to the identification of domains in p47<sub>phox</sub> mediating binding to p67<sub>phox</sub>. This was done by quantifying, by enzyme-linked immunosorbent assay, the binding of p67<sub>phox</sub> in solution, to a series of 95 overlapping biotinylated p47<sub>phox</sub> peptides, attached to streptavidin-coated 96-well plates. A single proline-rich domain (residues 357−371) was found to bind p67<sub>phox</sub> in the absence and presence of lithium dodecyl sulfate.

Phagocytic cells produce, in response to appropriate stimuli, a variety of oxygen-derived toxic radicals, all of which are derived from superoxide (O<sub>2</sub><sup>−</sup>). O<sub>2</sub><sup>−</sup> is generated by the NADPH-derived one-electron reduction of molecular oxygen, catalyzed by a membrane-associated heterodimeric flavocytochrome (cytochrome b<sub>559</sub>), composed of a 91-kDa glycoprotein (gp91<sub>phox</sub>) and a 22-kDa protein (p22<sub>phox</sub>), and incorporating two redox centers, FAD and two hemes (reviewed in Refs. 1−3). The conversion of cytochrome b<sub>559</sub> from the resting to the activated state is, most likely, the result of a conformational change leading to a high turnover electron flow from NADPH to oxygen, through the redox centers. This change in conformation is brought about by protein-protein interactions involving the two cytochrome b<sub>559</sub> subunits and four cytosolic regulatory proteins, p47<sub>phox</sub>, p67<sub>phox</sub>, p40<sub>phox</sub>, and the small GTPase Rac (1 or 2) (reviewed in Refs. 4 and 5). It is commonly assumed that activation leads to the formation of a membrane-localized multimolecular structure, known as the NADPH oxidase complex. The physical expression of this, in the intact cell, is the translocation of parts of p47<sub>phox</sub> and p67<sub>phox</sub> to the plasma membrane (6) associated with the phosphorylation of p47<sub>phox</sub> at multiple sites (7). Whether the activation of NADPH oxidase involves translocation of p40<sub>phox</sub> and Rac to the membrane is as yet an unsettled question.

The conversion of cytochrome b<sub>559</sub> from the inactive to the active conformation can be achieved in vitro in a cell-free system, consisting, in its most elementary form, of membranes and cytosol from resting cells exposed to a critical concentration of certain fatty acids (8−11) or anionic amphiphiles (12). An advanced version of the cell-free system, consisting of solubilized membrane or purified and lipid-reconstituted cytochrome b<sub>559</sub> combined with purified recombinant cytosolic components (13, 14), served as a valuable tool for the analysis of intermolecular interactions in the assembly of NADPH oxidase. p47<sub>phox</sub> plays a pivotal role in the activation of NADPH oxidase, so far it is the only cytosolic component for which unequivocal evidence is available for physical binding to cytochrome b<sub>559</sub> (reviewed in Ref. 4).

A number of methodologies have been utilized in the past for the detection of protein-protein interactions between p47<sub>phox</sub> and other NADPH oxidase components and for the identification of domains in the amino acid sequence of p47<sub>phox</sub> essential for its participation in the assembly of the complex (reviewed in Ref. 5). We recently described a new approach to mapping functionally significant domains in proteins, based on the use of overlapping peptides spanning the complete sequence of the protein under scrutiny. This methodology, termed “peptide walking,” was applied to the analysis of Rac1 domains involved.

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<sup>1</sup>The abbreviations used are: O<sub>2</sub><sup>−</sup>, superoxide; Tween 20, polyoxyethylene sorbitan monolaurate; LiDS, lithium dodecyl sulfate; GTPγS, guanosine 5′-3-(thio)triphosphate; PBS, phosphate-buffered saline; SH3, src homology 3; BSA, bovine serum albumin.
in the activation of NADPH oxidase (15). In the present paper we applied peptide walking to the analysis of p47\(^{\text{phox}}\) domains involved in the activation of NADPH oxidase. This research had two goals as follows: 1) evaluate the reliability of the method by its ability to detect domains identified before by other approaches, and 2) assess the capacity of the method to identify previously unknown domains. Finally, peptide walking was used as a method to identify domains in p47\(^{\text{phox}}\) participating in interaction with p67\(^{\text{phox}}\).

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**

**Synthetic Peptides—**Ninety-five overlapping pentapeptides spanning the complete p47\(^{\text{phox}}\) amino acid sequence, from the N to the C terminus, were synthesized by the multipin synthesis method (16) by Chiron Mimotopes, Clayton, Australia. Peptides overlapped by 11 residues, with the exception of peptides 94 and 95 which shared 12 residues. Peptides were biotinylated at the N terminus and were amimidated at the C terminus. The biotin group was attached to the N terminus by the intermediary of a four-residue spacer consisting of SGSG. The purity of the peptides ranged from 60 to 70%. The freeze-dried peptides were dissolved in a mixture of 75% dimethyl sulfoxide and 25% parts water (v/v), to a concentration of 1.5 mg, divided into aliquots of 50 μl and frozen at -75 °C.

**Chemicals**—The following chemicals were obtained from Sigma: ferricytochrome c (from horse heart, 95%), NADPH (tetrosodium salt, 95%), bovine serum albumin (crystallized, >97%; BSA), 3,3',5,5'-tetramethylbenzidine, free base, poloxonyethylene sorbitan monolaurate (Tween 20), streptavidin (from Streptomyces avidinii, affinity purified), and ImmunoProbe biotinylation kit (product number BK-101). Guanosine 5'-3'-(thio)triphosphate (GTP\(^\gamma\)S) was obtained from Boehringer Mannheim. N-Octyl-β-D-glucopyranoside (octyl glucoside) was a product of Pfaltzstiel. Lithium dodecyl sulfate (LiDS) and common laboratory supplies were used as required.

**Antibodies—**A polyclonal antibody against purified baculovirus-derived recombinant p67\(^{\text{phox}}\) was prepared in goats (17) and kindly provided, as whole serum, by Dr. Thomas L. Leto (National Institutes of Health, Bethesda, MD). Rabbit polyclonal antibody to BSA (whole serum) was obtained from Sigma. Peroxidase-conjugated affinity purified rabbit anti-goat IgG and goat anti-rabbit IgG were purchased from Jackson ImmunoResearch.

**Preparation of Recombinant Cytosolic NADPH Oxidase Components—**Recombinant p47\(^{\text{phox}}\) and p67\(^{\text{phox}}\) were prepared from baculovirus-infected Sf9 cells, as described by Leto et al. (17), and purified by ion exchange chromatography, as recently described (18). Recombinant Rac1 was isolated from E. coli transformed with Rac1 cDNA subcloned into the bacterial expression vector pGEX2T, as described by Kwong et al. (19). The glutathione S-transferase-Rac1 fusion protein was affinity purified using immobilized glutathione-agarose, cleaved by thrombin, and subjected to nucleotide exchange to GTP\(^\gamma\)S, as described before (20). Baculoviruses carrying cDNA for p47\(^{\text{phox}}\) and p67\(^{\text{phox}}\) and Rac1 cDNA subcloned in the pGEX2T vector were kind gifts of Dr. Thomas L. Leto (National Institutes of Health, Bethesda, MD).

**Biotinylation of p47\(^{\text{phox}}\)**

Purified recombinant p47\(^{\text{phox}}\) (at a protein concentration of 0.5 mg/ml) was biotinylated with biotinamidoacaproic acid, using the ImmunoProbe kit (Sigma) and following the instructions provided with the kit. The biotin/protein ratio varied from 3 to 3.5 mol of biotin per mol of p47\(^{\text{phox}}\).

**Preparation of Solubilized Macrophage Membranes**

Macrophages were obtained from the peritoneal cavity of guinea pigs injected with mineral oil 5–6 days earlier. Membranes were prepared from cells disrupted by sonication, as reported before (21), but using a modified homogenization buffer, which consisted of 130 mM NaCl, 5 mM Na\(^+\), potassium phosphate buffer, pH 7.4, 500 μM succrose, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM dithioerythritol, and 2 mM NaN\(_3\) (resulting in a final concentration of 0.3 mM LiDS). After removal of washing buffer, nonspecific absorption was blocked by adding to each well 200 μl of 10% (v/v) liquid milk (1% fat) in PBS and shaking the plates for 1 h at room temperature. The plates were washed four times with PBS-T, and to the wells were added 100-μl volumes of biotinylated p47\(^{\text{phox}}\) peptides or biotinylated p47\(^{\text{phox}}\).
protein, dissolved in 10% milk in PBS, to result in 50 pmol of peptide or protein per well. Binding of biotinylated compounds to the streptavidin-coated plates was allowed to proceed for 30 min at room temperature, with shaking. The plates were again washed four times with PBS-T and, following the removal of washing buffer, 100-ml volumes p67phox, dissolved in 10% milk in PBS, were added, to result in 50 pmol of protein per well. The plates were shaken for 30 min at room temperature to allow the interaction between the surface-attached p47phox peptides or p47phox protein and p67phox, in solution, to proceed. In control experiments, p67phox was replaced by 50 pmol/well of BSA. Unbound protein was removed by washing the plates four times with PBS-T, and the specifically bound p67phox and nonspecifically bound BSA were detected by the addition of 100-ml/well of goat anti-p67phox and rabbit anti-BSA antisera, diluted 1/1000 in 10% milk in PBS, respectively. The plates were incubated with the antibodies for 30 min at room temperature, with shaking, and unbound antibody was removed by four washes with PBS-T. Bound immunoglobulin was detected by the addition of a 1/10,000 dilution, in 10% milk in PBS, of peroxidase-conjugated anti-goat and anti-rabbit IgG antibodies, respectively, and incubation for 30 min at room temperature, with shaking. The plates were washed four times with PBS-T and once with distilled water, and bound peroxidase was detected by the addition of 100-ml/well of 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine and 0.006% H2O2 in 0.1 M sodium acetate buffer, pH 5.4. After incubation for 30 min at room temperature, the reaction was stopped by the addition of 50-ml/well of 2 N H2SO4. Absorbance at 450 nm was read against blank wells, containing only the peroxidase assay reagent, in a Spectramax 340 microplate reader (Molecular Devices).

**RESULTS**

Clusters of Overlapping p47phox Peptides Inhibit NADPH Oxidase Activation in a Cell-free Assay—Ninety five penta-decapeptides spanning sequentially the complete amino acid sequence of p47phox, starting at the first 15 residues at the N terminus and overlapping by 11 residues, were tested for an effect on the activation of NADPH oxidase in a cell-free system, consisting of solubilized macrophage membrane and recombinant p47phox, p67phox, and Rac1-GTPGS. Fig. 1 lists the peptides utilized in these experiments and the location of the corresponding sequences within the p47phox protein.

The assay conditions were subject to strict standardization. These included a fixed concentration of cytochrome b559 of 10 nM; concentrations of p67phox and Rac1 of 200 nM, corresponding to saturating levels in dose-response curves; and a concentration of p47phox of 60 nM, in the linear portion of a dose-response curve performed in the presence of the indicated concentrations of p67phox and Rac1. This concentration of p47phox was selected with the purpose of maximizing any competitive inhibitory effect by p47phox peptides, added at a constant concentration of 30 nM. Also, the order of addition of the various components of the reaction was designed to favor the interaction of p47phox peptides, over that of intact p47phox, with
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Fig. 2. Inhibition of NADPH oxidase activation by overlapping p47\textsuperscript{phox} pentadecapeptides. Peptides were assayed for the ability to inhibit O\textsubscript{2}\textsuperscript{-} production in an amphiphile-activated cell-free system consisting of solubilized macrophage membrane and recombinant p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac1-GTP\textsuperscript{S}. Peptides were added, at a concentration of 30 μM, either before the induction of activation (A) or 90 s after the induction of activation (B). Bars represent means ± S.E. from six (A) or three (B) experiments. Letters a–h indicate the nomenclature of the domains and are placed next to the cluster of inhibitory peptides, which led to the definition of the respective domain.

By using this strategy, we mapped 10 putative functional domains in the primary structure of p47\textsuperscript{phox}, which appear to participate in the activation of NADPH oxidase. These are pointed out in Fig. 1, by emphasizing the relevant residues, in each cluster of inhibitory peptides, by bold lettering, and by a marginal marking, in bold italics, of the nomenclature of the peptide clusters. Fig. 3 illustrates the location of the proposed domains in the sequence of the p47\textsuperscript{phox} protein, the labeling of the domains paralleling that of the peptide clusters in Figs. 1 and 2.

We next determined the IC\textsubscript{50} for selected individual peptides belonging to 7 of 10 peptide clusters. From each group, a peptide was chosen which caused maximal inhibition of NADPH oxidase activation when tested at a concentration of 30 μM. As evident in Fig. 4, peptides representative of clusters a–f had IC\textsubscript{50} values ranging from 8.5 to 11.5 μM; peptide 325–339, belonging to cluster g, had the lowest IC\textsubscript{50} (3.4 μM). These values are in the range or somewhat lower than those found for inhibition of NADPH oxidase activation by Rac1 peptides (15).

The most likely explanation for the inhibition of oxidase activation by p47\textsuperscript{phox} peptides was that these competed with intact p47\textsuperscript{phox} for a target protein among NADPH oxidase components. One approach to verifying the competitive nature of the effect of peptides on NADPH oxidase activation is to perform a kinetic analysis of the inhibition. This was used by us before to show that the inhibitory effect of polybasic C-terminal Rac1 undecapeptides was noncompetitive with respect to Rac1 (29; see also contradictory results in Ref. 30). We, therefore, performed a Michaelis-Menten analysis of the influence of varying the concentration of p47\textsuperscript{phox} (15–40 nM) on the inhibitory effect of selected peptides (representative of the various clusters) on NADPH oxidase activation. As shown by the Lineweaver-Burk plots in Fig. 5, illustrating the results obtained with peptide 325–339 (cluster g), the peptide behaves as a competitive inhibitor with respect to p47\textsuperscript{phox} protein. Similar
putative functional domains in the amino acid sequence of p47phox involved in NADPH oxidase assembly and binding of p67phox. Amino acids in **boldface** represent domains deduced by inhibition of NADPH oxidase activation. Amino acids in **boldface italic with underline** represent the domain binding p67phox. The position of each domain in the p47phox sequence and its nomenclature is indicated on the top half of the figure and the boundaries of each domain, by N- and C-terminal residue numbers, are shown in the bottom half.

**Table 1.** Putative functional domains in the amino acid sequence of p47phox involved in NADPH oxidase assembly and binding of p67phox.

| Domain | Residues |
|--------|----------|
| a₁     | 5 - 19   |
| a      | 21 - 35  |
| a₂     | 37 - 51  |
| b      | 105 - 119|
| c      | 149 - 159|
| d      | 193 - 207|
| e      | 253 - 267|
| f      | 305 - 319|
| g      | 325 - 339|
| h      | 357 - 371|
| PE     | 373 - 387|

p67phox binds to surface-immobilized p47phox and to a single cluster of p47phox peptides—The first purpose of the experiments described in this section of the study was the design of a simple, quantitative, and generally applicable methodology for assessing protein to protein binding among components of the NADPH oxidase complex. We here describe its use in the study of the interaction between p47phox and p67phox. This model was chosen based on evidence, originating from several groups of investigators, that p47phox and p67phox bind to each other constitutively to form a complex of a Mₐ of 240–300,000 (40–43) and on our recent results showing the formation of a stable complex between highly purified recombinant p47phox and p67phox, upon gel filtration of a mixture of the two components.²

With the purpose of identifying domains in p47phox involved in binding of p67phox, we made use of a technique originally developed for identifying linear epitopes in protein sequences recognized by antibodies. This consists of attaching multiple overlapping biotinylated peptides, spanning the sequence of the protein antigen, to streptavidin-coated 96-well plates, followed by reacting these with the antibody under study in the fluid phase (44). Therefore, we attached the 95 overlapping biotinylated p47phox pentadecapeptides (used before in the inhibition of NADPH oxidase activation experiments) and intact biotinylated p47phox protein to 96-well plates, previously coated with streptavidin, as described under "Experimental Procedures." Addition to the plates of p67phox, in the fluid phase, resulted in its binding to intact p47phox and to 2 out of 95 peptides (peptides 357–371 and 361–375) (Fig. 6A). In control experiments, it was found that BSA did not bind to either surface-attached intact p47phox or to any of 95 surface-attached p47phox peptides (Fig. 6B). As apparent from the list of peptides in Fig. 1, the two peptides binding p67phox shared a domain extending from residues 357 to 371, which was labeled pr (proline-rich) to indicate the presence of five proline residues. The location of domain pr in the p47phox sequence is indicated in Fig. 3. It is of interest that peptides 357–371 and 361–375 were found not to inhibit NADPH oxidase activation (Fig. 2).

We next investigated the specificity of binding of p67phox, in the fluid phase, to the surface-attached proline-rich p47phox peptides. This was done by preincubating 50–pmol amounts of p67phox with intact p47phox (5–100 pmol) in solution for 15 min at room temperature, before addition to 96-well plates coated with either peptide 357–371 or 361–375. As a control, 50 pmol of p67phox was preincubated with 100 pmol of BSA. As apparent in Fig. 7, preincubation of p67phox with p47phox led to a dose-dependent reduction in binding of p67phox to both proline-rich p47phox peptides, reaching 96–97% inhibition of binding at 100 pmol p47phox; preexposure to BSA did not interfere with binding to the peptides.

We also examined whether domains in p47phox, interacting with p67phox, are revealed by performing the "p47phox peptides-p67phox protein" binding assay in the presence of the activating amphiphile LiDS. In these experiments, p67phox (or control BSA) was mixed with 130 μM LiDS in PBS supplemented with 2% (v/v) milk immediately before addition to the 96-well plates coated with p47phox peptides and intact p47phox, as a control. All other experimental conditions were as described for binding of p67phox in the absence of LiDS. We found that, in the presence of LiDS, p67phox bound normally to surface-attached p47phox and to the two peptides (357–371 and 361–375), which also bound p67phox in the absence of LiDS (results not shown). The extent of binding to these was identical to that measured in the absence of LiDS. Therefore, exposure to LiDS did not unmask sites in p67phox capable of binding to domains in p47phox, other than the one detected in the absence of LiDS.
In the present report we describe the application of peptide walking to the identification of domains, in the linear amino acid sequence of p47\textsuperscript{phox}, which participate in the assembly of an enzymatically active NADPH oxidase complex \textit{in vitro}. We used this methodology before for the definition of oxidase activation-related domains in Rac1 (15). Although overlapping peptides were tested exclusively as inhibitors of NADPH oxidase activation in this earlier report, we have now extended their use to binding studies involving p47\textsuperscript{phox} peptides and intact p67\textsuperscript{phox}.

The assumptions underlying the use of peptide walking for the identification of domains in p47\textsuperscript{phox} involved in the activation of NADPH oxidase were the following. 1) Inhibition of activity by peptides containing part of or a complete domain is the result of competition between peptide and p47\textsuperscript{phox} protein for a common target, and proof for this is provided by results of Michaelis-Menten analysis, demonstrating a competitive inhibition pattern with respect to p47\textsuperscript{phox}. 2) The use of overlapping peptides results in a high likelihood for a domain or part of it to be present in several neighboring peptides, assuring more sensitive and reliable detection than that offered by the use of selected individual peptides. 3) Peptide walking is, by definition, detecting domains involved in NADPH oxidase activation in the cell-free system, and their role in the elicitation of an oxidative burst in whole cells has to be evaluated by additional methods.

The main goal of this research was to assess the capacity of peptide walking to serve as a screening method for the identification of functional domains in p47\textsuperscript{phox}. The success of this methodology was demonstrated by its ability to both detect domains identified before by other techniques and to reveal novel domains. Thus, we identified eight domains (11–15 residues) in the linear sequence of p47\textsuperscript{phox} participating in NADPH oxidase activation \textit{in vitro}. Four of these correspond to regions identified previously by other methods (domains c–e and g), and four represent newly identified domains (a, b, f, and h) for which no experimental evidence was available for involvement in activation. The reality of two additional domains (a1 and a2), located in the vicinity of domain a, based solely on the inhibitory action of single peptides will have to be confirmed by other methods.

Domain a spans residues 21–35 and is rich in hydrophobic amino acids. It does not share residues with domains a1 and a2, but the possibility cannot be excluded that we are dealing with a larger region composed of all three nearly contiguous modules. Recently, it was found that the N-terminal regions of p47\textsuperscript{phox} and p40\textsuperscript{phox} contain a previously unidentified domain, termed PX, also shown to be present in the Cpk class of phosphatidylinositol 3-kinase and in several yeast proteins (Bem1p and Scd2) (45). In p47\textsuperscript{phox}, domain PX was proposed to span residues 20–125, and it is possible that the domain might also include a region N-terminal to residue 20 (45). It is of interest that domains a and a2 are located within the limits of the PX region and contain no less than six PX consensus residues. Should the PX domain extend further toward the N terminus, it might possibly also include domain a1. Potential ligands for PX domains are SH3 domains and, putatively, Rho-type GTPases (46). The PX region also incorporates domain b (residues 105–119), which contains two PX consensus residues (phenylalanines 117 and 118). It should be noted that the PX domain...
was proposed as a new entity exclusively on the basis of a sequence similarity search, and no experimental proof existed for its participation in NADPH oxidase activation. Therefore, it appears that the N-terminal region of p47\textsuperscript{phox}, extending from residue 5 to 119, is required for O\textsubscript{2}\textsuperscript{−} production in a semi-recombinant cell-free system. The only evidence in the literature in support of this is the finding that O\textsubscript{2}\textsuperscript{−} production by K562 cells transfected with a vector containing cDNA coding for p47\textsuperscript{phox}, lacking the 150 N-terminal residues, was reduced by 65% in comparison to cells transfected with full-length p47\textsuperscript{phox} cDNA (47).

Domain c spans residues 149–159 and is adjacent to the N-terminal limit of the first SH3 domain. A region extending from residue 151 to 155 was found to be required for O\textsubscript{2}\textsuperscript{−} production in transfected K562 cells and for binding of the N-terminal SH3 domain of p47\textsuperscript{phox} to p22\textsuperscript{phox} in vitro and in whole cells (47, 48). Some authors (5) consider it as merely part of the N-terminal SH3 domain, and the fact that it was highlighted by peptide walking supports the contention that it represents an independent domain.

Domain d spans residues 193–207 and is located in the C-terminal half of the N-terminal SH3 domain of p47\textsuperscript{phox}. p47\textsuperscript{phox} contains two SH3 domains (residues 161–212 and 231–281) (49, 50), and these were found to play a key role in the assembly of the NADPH oxidase complex (33, 35, 47, 48). It has been reported that the N-terminal SH3 domain is alone responsible for binding to the p22\textsuperscript{phox} subunit of cytochrome b\textsubscript{559} and participates in an intramolecular interaction with the C-terminal proline-rich domain of p47\textsuperscript{phox} (residues 360–370) (48, 51). Both claims were recently contradicted on the basis of their reexamination by three binding assays, applied in parallel (52).

Peptide walking revealed a subdomain, within the 60-amino.
acid long N-terminal SH3 domain of p47\textsubscript{phox}, which appears to be of special importance in NADPH oxidase assembly. SH3 domains are unique protein-protein signaling motifs, the target of which are proline-rich sequences, adopting a polyproline type II helix conformation (reviewed in Ref. 53). SH3 domains make contact with these ligands via three hydrophobic sites; the first two sites (S1, S2) contain highly conserved residues interacting with a universal PXXP motif, whereas the third site (S3) contains nonconserved residues and determines target specificity. Domain d contains Trp-193 and Pro-206, two conserved residues found in the S2 site, and Trp-204, a nonconserved residue in the S3 site. Trp-193 is one of the strictly conserved residues in SH3 domains (51); a Trp-193 \rightarrow Arg mutation resulted in the loss of the ability of the mutant protein to support O\textsubscript{2}\textsuperscript{-} production in the transfected K562 cell (48) and cell-free (51) models. Also, Pro-206 \rightarrow Leu and Trp-204 \rightarrow Ala mutations resulted in the total abolishment of O\textsubscript{2}\textsuperscript{-} production in transfected K562 cells. It is significant that peptide 193–207, which exerted the most potent inhibition of oxidase activation among peptides of cluster d (94.4 \pm 1.8%), also contained all three essential residues.

Domain e spans residues 253–267 and is located close to the center of the C-terminal SH3 domain. It was defined on the basis of the moderate oxidase activation inhibitory activity of a single peptide (253–267). The domain contains Trp-263, a highly conserved residue in all SH3 domains (54) which forms part of the S2 site. Indeed, K562 cells transfected with a Trp-263 \rightarrow Arg peptide walking exhibit a partial reduction in O\textsubscript{2}\textsuperscript{-} production (48).

It is remarkable that the two SH3 subdomains emphasized by peptide walking contain five out of the seven tryptophans present in p47\textsubscript{phox}, including three functionally essential tryptophans (Trp-193, Trp-204, and Trp-263). In addition, a sixth tryptophan is located in domain d, discussed above. Recently Quinn and colleagues (55) have reported that treatment of p47\textsubscript{phox} with oxidase-activating concentrations of SDS or arachidonate, as well as protein kinase C-mediated phosphorylation of p47\textsubscript{phox}, cause quenching of intrinsic tryptophan fluorescence, indicative of a conformational change. It is tempting to speculate that at least some of the tryptophans situated in the domains revealed by peptide walking are involved in protein-protein interactions related to NADPH oxidase activation.

Domains f (residues 305–319) and g (residues 329–339) were defined by the oxidase inhibitory activity of two clusters of peptides rich in basic amino acids and of low hydrophobicity. The boundary separating the two domains is arbitrary, and it is possible that we are dealing with a single large domain spanning residues 301–339, containing a high proportion (38.46%) of basic residues, principally arginine. A functionally important region within p47\textsubscript{phox} encompassing residues 323–332 (56) or 324–331 (57), was described on the basis of inhibition, by selected peptides, of O\textsubscript{2}\textsuperscript{-} generation and translocation of p47\textsubscript{phox} to the membrane in a cell-free system. A region extending from residues 323 to 342 was also identified from a random phage display library, by biopanning with cytochrome b\textsubscript{552} (58) and p67\textsubscript{phox} (59), suggesting that it serves as a binding site in p47\textsubscript{phox} for both cytochrome b\textsubscript{552} and p67\textsubscript{phox}, probably at successive steps in the process of oxidase assembly. Domain f, as such, was not reported before as an independent, functionally important region, and whether it represents a distinct domain will have to be established by additional methods. A p47\textsubscript{phox} deletion mutant lacking residues 236–350, which includes domains e, f, and g, showed a markedly reduced capacity to support O\textsubscript{2}\textsuperscript{-} production in a cell-free system (60). The positively charged region extending from residue 303 to 379 also contains all the serines phosphorylated in the course of phagocyte activation leading to an oxidative burst (61). The cationic nature of the peptides inhibiting NADPH oxidase activation, on which definition of domains f and g was based, raises the issue of a possible nonspecific, charge-mediated effect. Indeed, we have reported sequence-unrelated inhibition of NADPH oxidase activation by basic homopolymers (29). The positive charge of the C terminus of p47\textsubscript{phox} is considered to be of functional significance in the interaction of p47\textsubscript{phox} with other oxidase components and may serve as the target for neutralization by phosphorylation in vivo or anionic amphiphile action in vitro (58, 61). However, inhibition of NADPH oxidase activation by cluster f and g peptides appears to be sequence-specific, as indicated by Michaelis-Menten analysis demonstrating competitive inhibition with respect to p47\textsubscript{phox}. Furthermore, earlier work involving the comparison of the oxidase inhibitory effects of selected peptides, broadly corresponding to domain g, with peptides containing a similar proportion of basic residues, showed convincingly that charge was not a major determinant of inhibition (56–58).

Domain h spans residues 373–387, close to the C terminus of the protein. There is no previous description of this domain in p47\textsubscript{phox} as being essential for NADPH oxidase activation; thus, p47\textsubscript{phox} truncated at residue 367 exhibited normal oxidase-activating ability in the cell-free system (60). It has been reported that preincubation of p47\textsubscript{phox} with an antibody to residues 379–390, or deletion of residues 378–390, leads to the loss of the ability of native p47\textsubscript{phox} to bind to p67\textsubscript{phox} (60). It is of potential significance that the domain contains Ser-379, the phosphorylation of which appears to be of key importance for oxidase assembly (62). Also, a peptide partly overlapping domain h (residues 378–390) could serve as a substrate for phosphorylation by protein kinase C and by a kinase present in neutrophil cytosol (63). Therefore, it is possible that the stretch of amino acids surrounding Ser-379 might be an additional target for an activation related conformational change, with LiDS mimicking in vitro the phosphorylation occurring in the intact phagocyte.

The second part of our work describes the use of peptide walking for identifying sites of interaction on p47\textsubscript{phox} with the cytosolic protein, p67\textsubscript{phox}. For this purpose, biotinylated p47\textsubscript{phox} peptides were attached to streptavidin-coated 96-well plates, and p67\textsubscript{phox} was added in solution. By applying this method, a single p67\textsubscript{phox}-binding domain was identified, corresponding to residues 357–371 (domain pr). Binding to p47\textsubscript{phox} peptides was specific for p67\textsubscript{phox}, amphiphile-independent, and was competed for by intact p47\textsubscript{phox} protein. No additional p67\textsubscript{phox}-binding domains were revealed by performing the assay in the presence of the oxidase activating amphiphile, LiDS. The principal candidate, among NADPH oxidase components, for the role of amphiphile target was thought to be p47\textsubscript{phox} (32, 35, 55), but recent evidence indicates that both p47\textsubscript{phox} and p67\textsubscript{phox} are subject to unfolding by anionic amphiphiles (64). This is in agreement with earlier indirect evidence for an effect of amphiphiles on p67\textsubscript{phox} (32, 65, 66). The design of our binding assay did not allow the investigation of the effect of LiDS on p47\textsubscript{phox} since p47\textsubscript{phox} was replaced by a series of peptides which, by definition, represent the “unfolded” state of the molecule. Therefore, the likely target of LiDS in the binding assay would have been the native component, p67\textsubscript{phox}. Our results, however, show that binding of p67\textsubscript{phox} to domain pr on p47\textsubscript{phox} is not dependent on or influenced by amphiphile, indicating that unfolding of p67\textsubscript{phox} is not a prerequisite for the establishment of this particular bond. Although the effect of amphiphile on p47\textsubscript{phox} could not be explored in our experiments, results derived from the two-hybrid system indicate that unfolding of
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p47<sub>phox</sub> is also not required for exposure of domain pr and binding of p67<sub>phox</sub> to it (64).

Domain pr, revealed by the binding assay, corresponds closely to the C-terminal proline-rich motif, shown to interact with the C-terminal SH3 domain in p67<sub>phox</sub> (31, 35, 47, 67, 68). This tail-to-tail interaction is not involved in NADPH oxidase activation in the cell-free system. Thus, it was shown that deletion mutants of p47<sub>phox</sub>, lacking part of the proline-rich domain, were unable to bind p67<sub>phox</sub> but were, nevertheless, capable of supporting O<sub>2</sub><sup>-</sup> production in the cell-free system (60). Reciprocally, p67<sub>phox</sub> deletion mutants, lacking the C-terminal SH3 domain and therefore incapable of binding to the proline-rich domain of p47<sub>phox</sub>, were capable of supporting O<sub>2</sub><sup>-</sup> production in the cell-free system (64, 68, 69). This explains the fact that domain pr was not detected by the inhibition of NADPH oxidase activity assay. Our conclusions concerning the lack of involvement of domain pr in cell-free oxidase activation might not apply to events in the intact cell. Work in the transfected cell model, indeed, suggests that tail-to-tail interactions between p47<sub>phox</sub> and p67<sub>phox</sub> might contribute to oxidase activation by optimizing p67<sub>phox</sub> recruitment to the membrane (47, 70).

The binding assay, whether performed in the absence or presence of LiDS, did not reveal the binding of p67<sub>phox</sub> via its N-terminal proline-rich domain, to the C-terminal SH3 domain of p47<sub>phox</sub>. This interaction was reported to occur under NADPH oxidase activating conditions (32, 47, 48). A likely explanation for this failure is that binding of the relatively small proline-rich motif, in p67<sub>phox</sub>, to the large C-terminal SH3 domain in p47<sub>phox</sub> involves several ligand binding sites, separated by distances exceeding 15 residues and requiring the conservation of secondary structure. The assay also failed to demonstrate binding of p67<sub>phox</sub> to domain g, identified by inhibition of oxidase activation, and was recently reported as a p67<sub>phox</sub>-binding region on the basis of random peptide phage-display analysis of p67<sub>phox</sub> (59). The reason for this failure could not be established.

In conclusion, overlapping biotinylated peptides, spanning the complete p47<sub>phox</sub> sequence, proved to be effective tools for the identification of domains participating in NADPH oxidase activation. This is shown by the ability of the technique to (a) confirm the existence of domains identified earlier by other methodologies, (b) narrow down the borders of domains described previously, and (c) identify novel domains. The use of peptide walking for the identification of domains in p47<sub>phox</sub> involved in binding p67<sub>phox</sub> was only partially successful. Thus, the method failed to detect two domains, identified by other methodological approaches.

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