Properties of phagocyte NADPH oxidase p47-phox mutants with unmasked SH3 (Src homology 3) domains: full reconstitution of oxidase activity in a semi-recombinant cell-free system lacking arachidonic acid

Guihong PENG, Jin HUANG, Mellonie BOYD and Michael E. KLEINBERG[1]

Research Service, Veterans Affairs Maryland Health Care System-Baltimore and Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A.

In an early step in the assembly of the phagocyte NADPH oxidase, p47-phox translocates from the cytosol to the membrane, mediated by engagement of the N-termini of two p47-phox Src homology 3 (SH3) domains with a proline-rich region (PRR) in the p22-phox subunit of cytochrome b_{558}. In response to phagocyte activation, several serine residues in a C-terminal arginine/lysine-rich domain of p47-phox are phosphorylated, leading to changes in the conformation of p47-phox and exposure of its N-terminal SH3 domain that is normally masked by internal association with the arginine/lysine-rich domain. We report that triple alanine substitutions at Asp-217, Glu-218 and Glu-223 in a short sequence that links the tandem p47-phox SH3 domains unmasked the N-terminal SH3 domain, similar to the effects of aspartic acid substitutions at Ser-310 and Ser-328 in the arginine/lysine-rich region. Recombinant p47-phox proteins with mutations in either the linker region or the arginine/lysine-rich domain were active in the absence of arachidonic acid stimulation in a cell-free NADPH oxidase system consisting of recombinant p67-phox, Rac1–guanosine 5′-[γ-thio]triphosphate and neutrophil membranes. Supplementing neutrophil membranes with phosphoinositides or other negatively charged phospholipids markedly enhanced cell-free superoxide generation by these p47-phox mutants in the absence of arachidonic acid, to levels equivalent to those generated by wild-type p47-phox following arachidonic acid activation. This enhancement may be related to recruitment to the membrane of p47-phox mediated by a novel secondary phox homology (PX) domain binding site that broadly recognizes phospholipids. No specific enhancement by specific phosphorylated phosphatidylinositols was found to suggest a dominant role for the p47-phox primary PX domain binding site. Truncated p47-phox S310D S328D lacking the C-terminal PRR was inactive in the cell-free system without arachidonic acid, but was fully active with arachidonic acid. This suggests that activation of NADPH oxidase in an arachidonate-free cell-free system requires association of the p47-phox C-terminal PRR with the p67-phox C-terminal SH3 domain.

Key words: NADPH oxidase, p47-phox, phox homology domain, proline-rich region, SH3 domain.

INTRODUCTION

The phagocyte NADPH oxidase complex is a critical component of the innate immune system defending against bacterial, fungal, and viral pathogens. NADPH oxidase catalyses the reduction of molecular oxygen to superoxide, which is then converted into other oxidants, including hydrogen peroxide, hydroxyl radical and hypohalous acids [1]. Oxidants generated by NADPH oxidase do not discriminate between microbial and host targets [2]. One important mechanism for controlling the formation of NADPH oxidase is to sequester its various component proteins in different cellular compartments, and to allow the oxidase to assemble only in response to specific signals that indicate that a pathogen has been encountered [1,3,4].

Integral membrane cytochrome b_{558} consists of the gp91-phox and p22-phox subunits in a 1 : 1 complex [5,6], and binds NADPH, FAD and two haems [7,8]. The catalytically active oxidase is formed after cytochrome b_{558} interacts with at least three cytosol proteins, i.e. p47-phox, p67-phox and Rac2 (or Rac1), which translocate to the membrane and complex with cytochrome b_{558} coincident with neutrophil activation [1,3,4]. GTP-bound Rac1 or Rac2, members of the Rho class of GTPases, binds p67-phox [9], which facilitates electron transfer via cytochrome b_{558} [10].

p47-phox possesses two Src homology 3 (SH3) domains [3]. The N-terminal SH3 domain (SH3A) (Figure 1a) binds to a proline-rich region (PRR) in the p22-phox C-terminus, and this interaction is essential for NADPH oxidase activity [11]. The function of the C-terminal SH3 domain (p47 SH3B) is less well understood, but it may bind a PRR in the p47-phox N-terminus [12,13]. This newly described PRR is adjacent to an N-terminal phox homology (PX) domain, which binds phosphatidylinositide phosphates (PI-Ps) [12–14]. Another PRR located in the p47-phox C-terminus binds an SH3 domain in the p67-phox C-terminus [15]. p40-phox is not required for catalytic activity, but facilitates oxidase assembly [16] by complexing p47-phox and p67-phox [17] and targeting these phox proteins to PI-P-enriched cellular membranes via an N-terminal PX domain [14].

A positively charged arginine/lysine-rich domain (Arg/Lys domain) is located in the p47-phox C-terminus between the SH3B domain and the C-terminal PRR (Figure 1a). This Arg/Lys domain contains multiple serines within amino acid sequences conforming to consensus phosphorylation sites [18]. Stimulation

Abbreviations used: Arg/Lys domain, arginine/lysine-rich domain; diOG, 1,2-di-oleoylglycerol; GFP, green fluorescent protein; GST, glutathione S-transferase; p47 SH3AB, truncated p47-phox encompassing the two SH3 domains plus the intervening linker sequence (residues 154–285); PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI-P, phosphatidylinositide phosphate; PI-3-P, phosphatidylinositol 3-phosphate; PI-4-P, phosphatidylinositol 4-phosphate; PI-3,4-P, phosphatidylinositol 3,4-bisphosphate; PRR, proline-rich region; PS, phosphatidylserine; PX, phox homology; SH3, Src homology 3; TBS, Tris-buffered saline.

[1] To whom correspondence should be addressed: University of Maryland Greenebaum Cancer Center, Rm N9E05, UMH, 22 South Greene St, Baltimore, MD 21201 U.S.A. (e-mail mkleinbe@umaryland.edu).

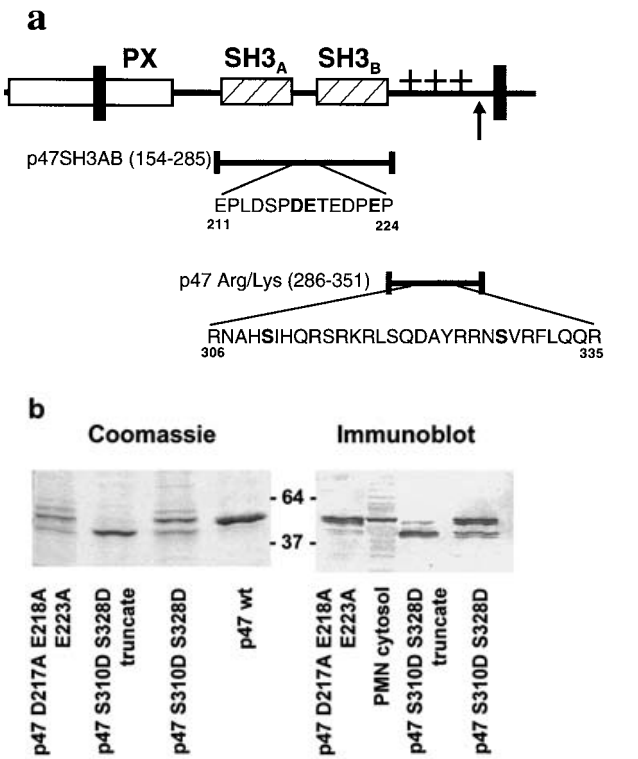

Figure 1 (a) Relative positions of structural motifs in the linear amino acid sequence of p47-phox, and (b) apparent molecular masses and immunoreactivities of recombinant p47-phox proteins

(**a**) N-terminal (SH3A) and C-terminal (SH3B) domains are indicated by hatched boxes. The N-terminal PX domain is shown by the solid white rectangle. N-terminal and C-terminal PRRs are depicted as vertical solid black bars. The p47-phox N-terminal PRR binds internally to the SH3B domain. The SH3A domain binds to a PRR in the p22-phox C-terminus. The p47-phox C-terminal PRR binds a C-terminal SH3 domain of p67-phox. The location of the positively charged Arg/Lys domain is marked by +++. Detailed amino acid sequences show the locations of the D217A E218A E223A mutations introduced to generate p47 D217A E218A E223A and p47 SH3AB D217A E218A E223A, and of the S310D S328D mutations introduced to generate p47 S310D S328D and p47 SH3AB S310D S328D (mutated amino acids are shown in bold). The arrow denotes the approximate location of the cleavage site that generates truncated p47 S310D S328D. (**b**) Left panel: recombinant p47 D217A E218A E223A (lane 1), p47 S310D S328D truncate (lane 2), p47 S310D S328D (lane 3) and wild-type (wt) p47-phox (lane 4) (each 4 μg/lane) were separated by SDS/PAGE on 4–20 % (w/v) acrylamide gradient gels. Protein bands were visualized with Coomassie Brilliant Blue. Right panel: recombinant p47 D217A E218A E223A (lane 1), p47 S310D S328D truncate (lane 3), p47 S310D S328D (lane 4) (each 2 μg/lane) and neutrophil (PMN) cytosol (lane 2; 63 μg of protein/lane) were separated by SDS/PAGE on 4–20 % gradient gels, transferred to a PVDF membrane and incubated with anti-p47-phox serum. Bands were visualized after further incubation with phosphatase-conjugated rabbit anti-goat IgG followed by colour development with 5-bromo-4-chloro-indolyl phosphate/NitroBlue Tetrazolium.

of neutrophils leads to the phosphorylation of multiple serines by one or more kinases, with the most heavily phosphorylated forms of p47-phox being bound to the membrane [18–20]. In unphosphorylated p47-phox, the Arg/Lys domain binds internally to the tandem SH3 domains [21,22]. This effectively prevents binding of the p47-phox SH3A domain to the p22-phox PRR, and p47-phox is confined largely to the cytosol. Phosphorylation of one or more serines in the Arg/Lys domain induces a conformational change that unmasks the p47-phox SH3A domain and enables it to bind to p22-phox [21,22]. The exact SH3 domain site that interacts with the Arg/Lys domain has not been identified. It has been suggested recently that this phosphorylation-dependent conformational change also unmasks a p47-phox N-terminal PX domain [13], which would be expected to allow binding to PI-P lipids and facilitate translocation from cytosol to membrane. In the present study, we used the NADPH oxidase cell-free system and binding assays to explore the effects of disrupted p47-phox Arg/Lys domain/SH3 domain binding on oxidase activity.

MATERIALS AND METHODS

Yeast two-hybrid binding assay

Saccharomyces cerevisiae EGY48, pEG202, pJG4-5 and pSH18-34 were generously donated by Roger Brent (Massachusetts General Hospital, Boston, MA, U.S.A.) and Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA, U.S.A.) [23]. cDNAs encoding the p22-phox C-terminus (residues 135–195), p47-phox and p67-phox were gifts from Thomas Leto (NIAID, NIH, Bethesda, MD, U.S.A.). cDNAs encoding full-length p47-phox (native sequence and alanine-substituted mutants), p47 SH3AB (a truncated p47-phox that encompasses the two SH3 domains plus the intervening linker sequence, i.e. residues 154–285); and alanine-substituted p47-phox variants p47 SH3AB D217A E218A and p47 SH3AB D217A E218A E223A (see Figure 1) were cloned into pJG4-5 and expressed as B42 activation domain fusion proteins. cDNA encoding p67-phox, p22-*phox* C-terminus (p22CT), and p47Arg/Lys (286–351) (Figure 1) were cloned into pEG202 and expressed as LexA binding domain fusion proteins [22]. Mutations in full-length p47-phox and p47 SH3AB were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) using native sequence full-length p47-phox and p47 SH3AB cDNAs respectively encoded in pGEX4T1 (Pharmacia, Piscataway, NJ, U.S.A.) as templates. Alanine substitutions at Asp-217 and Glu-218 were introduced at the same time into full-length p47-phox and p47 SH3AB using primer 5′-CCTGACGAGACGGC*AGC*CCCTGAGCCCAAC-3′, to produce full-length p47 D217A E218A and p47 SH3AB D217A E218A respectively. The E223A mutation was then introduced into full-length p47 D217A E218D and p47 SH3AB D217A E218A using primer 5′-GGAAGACCCTGC*GCCCAACTATG-3′. p47 D217A E218A E223A and p47 SH3AB D217A E218A E223A cDNAs were then cloned in-frame into pJG4-5 at 5′ *Eco*RI and 3′ *Sal*I sites. The correct sequences of all cDNAs were verified by automated DNA sequencing performed in the Biopolymer Laboratory at the University of Maryland, Baltimore. EGY48 was transformed with pJG4-5, pEG202 and pSH18-34 using lithium acetate as described [23]. Expression of LexA and B42 fusion proteins in EGY48 was confirmed by immunoblot analysis [22]. Prototrophy in leucine-deficient media indicated reconstitution of LexA transactivator function and transcription of the *LEU2* reporter [23].

Preparation of recombinant phox proteins

Recombinant Rac1 was expressed in *Escherichia coli* DH5α as a glutathione S-transferase (GST) fusion protein, purified and cleaved from GST with thrombin. Wild-type p47-phox and p67-phox were purified as described from baculovirus-infected Sf9 insect lysates [24]. Baculoviruses encoding p47 S310D S328D and p47 D217A E218A E223A were constructed using the Bac-to-Bac system (Life Technologies, Rockville, MD, U.S.A.) and expressed in Sf9 insect cells. Recombinant p47 S310D S328D and p47 D217A E218A E223A were purified by cation-exchange chromatography on DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) as described [24].

Full-length wild-type p47-phox and p47 S310D S328D ran as 51 kDa apparent molecular mass bands on 4–20 % (w/v) acrylamide gradient SDS/PAGE Tris/glycine gels (Invitrogen, Carlsbad, CA, U.S.A.) using Benchmark Protein Ladder prestained molecular mass markers (Invitrogen) (Figure 1b). Variable amounts of a single 46 kDa proteolytic fragment were seen with different preparations of recombinant p47 S310D S328D (Figure 1b). We have seen the same 5 kDa truncation of p47 S310D S328D with GST–p47 S310D S328D and green fluorescent protein (GFP)–p47-S310D S328D fusion proteins (results not shown). Since GST–p47 S310D S328D bound to glutathione–Sepharose and GFP–p47 S310D S328D was highly fluorescent, we surmised that the p47 S310D S328D lower-apparent-molecular-mass band represented a 5 kDa truncation from the C-terminus at a particularly sensitive proteolytic cleavage site. Truncated p47 S310D S328D eluted at lower ionic strength, allowing separation of the majority of the truncated from full-length p47 S310D S328D (Figure 1b). Using sonication instead of nitrogen cavitation to break Sf9 cells, in addition to withholding protease inhibitors, increased the percentage of truncated compared with full-length p47 S310D S328D. This simplified the purification of essentially pure truncated p47 S310D S328D (Figure 1).

Protein concentrations were measured with the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.) using BSA standards. Proteins were separated by SDS/PAGE on 4–20 % gels and transferred on to Immobilon P PVDF membranes (Millipore, Bedford, MA, U.S.A.). Membranes were blocked with 5 % (w/v) non-fat dried milk in Tris-buffered saline (TBS; 20 mM Tris/0.5 M NaCl, pH 7.5) for 1 h at room temperature. Membranes were incubated with goat anti-p47-phox serum (1:2500 dilution) for 1 h at room temperature, washed extensively, and incubated with phosphatase-conjugated rabbit anti-goat IgG (Kirkegaard and Perry, Gaithersburg, MD, U.S.A.; 1:5000 dilution) for 1 h at room temperature. Protein bands were visualized with 5-bromo-4-chloro-indolyl phosphate/NitroBlue Tetrazolium phosphatase substrate (Kirkegaard and Perry).

Cell-free assay for NADPH oxidase activity

The cell-free assay of superoxide generation was performed in 96-well microtitre plates in a final volume of 100 μl/well. Membrane was added to individual wells with a reaction mixture containing p67-phox (0.1–0.9 μM final concentration), Rac1 (0.2 μM final concentration), FAD (1 μM; Sigma, St. Louis, MO, U.S.A.), NADPH (200 μM; Sigma) and guanosine 5′-[γ-thio]triphosphate (10 μM; Sigma) [25]. Neutrophil membrane fractions were prepared as described [25]. In some experiments, phosphatidylinositol 3-phosphate (PI-3-P), phosphatidylinositol 4-phosphate (PI-4-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P) (Echelon Research Laboratories, Salt Lake City, UT, U.S.A.), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA) (Avanti Polar-Lipids, Alabaster, AL, U.S.A.), phosphatidylcholine (PC; egg lecithin) or 1,2-di-oleoylglycerol (diOG) (Sigma) in chloroform/methanol (2:1, v/v) was dried under nitrogen and then solubilized on ice with 1 % (v/v) deoxycholate for 30 min. Solubilized lipid was then added to neutrophil membranes at various ratios {expressed as a percentage, i.e. [(μg of lipid/(μg of lipid + μg of membrane protein)] × 100} followed by another 60 min of co-solubilization on ice. Lipid/membrane mixtures were then diluted 10-fold with water [0.1 % (w/v) deoxycholate and 100 μg/ml membrane protein]. A 10 μl aliquot of this lipid/membrane solution was added to each well (final amount of membrane protein = 10 μg/well).

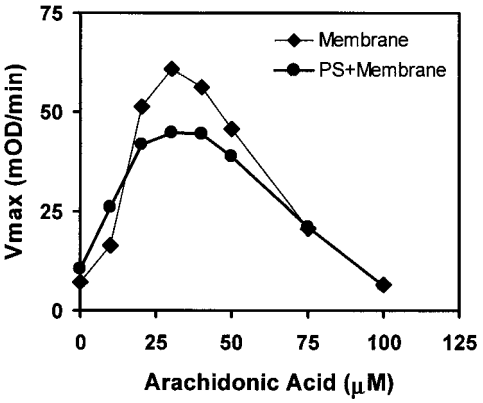

Figure 2 Dependence of NADPH oxidase activity on arachidonic acid concentration in the cell-free system

Neutrophil membranes (◆) or a 50 % PS/membrane mixture (●) (each 10 μg of protein/well) were incubated with recombinant p67-phox (0.9 μM), Rac1 (0.2 μM) and wild-type p47-phox (0.12 μM) and various concentrations of arachidonic acid in the cell-free system. V_{max} was measured as described in the Materials and methods section (OD = absorbance). Maximal NADPH oxidase activity was seen with 30 μM arachidonic acid. Shown are results from one of two identical experiments.

In some experiments, superoxide production was initiated by adding arachidonic acid (30 μM final concentration). This concentration of arachidonic acid maximally stimulates superoxide generation under cell-free experimental conditions used for both enriched and non-enriched neutrophil membranes (Figure 2). The maximal rate of superoxide formation (V_{max}) was obtained by calculating the maximal rate of superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma) monitored at 550 nm using a Molecular Devices (Menlo Park, CA, U.S.A.) Thermomax kinetic microtitre plate reader and Softmax (Molecular Devices) software [25]. We noticed somewhat greater day-to-day variability in NADPH oxidase activity of membrane preparations in cell-free experiments where arachidonic acid was not used compared with what we have observed when using arachidonic acid to stimulate cell-free NADPH oxidase activity. Variability was noted only in the magnitude of V_{max} (50–200 % range of variation), but results were otherwise qualitatively similar. Reported means and errors are from experiments repeated on the same day where the same membrane preparation was used. All experiments were repeated at least twice. p47-phox Arg/Lys domain peptide p47-(306–335) and corresponding phosphorylated peptide p47-(306–335) S310pS S328pS, as well as linker region peptides p47-(211–229) and p47-(211–229) D217A E218A E228A, were synthesized by the Biopolymer Laboratory at the University of Maryland, Baltimore. Final peptide purity was > 90 %, as estimated by HPLC. The correct sequences of synthesized peptides were confirmed by MS.

Blot binding assay

Aliquots of 20 pmol of full-length wild-type p47-phox, p47 S310D S328D and p47 S310D S328D truncate in TBS were spotted on to nitrocellulose (0.45 μm porosity) using a 96-well format manifold (Hybri-dot; Life Technologies) with gentle vacuum aspiration. The nitrocellulose membrane was blocked with SuperBlock Blocking Buffer (Pierce) for 5 min. Recombinant p67-phox (87 pmol in 200 μl of TBS) was added to the indicated wells and aspirated through the membrane

with vacuum. After washing, the nitrocellulose membrane was removed from the manifold and blocked with 5 % (w/v) non-fat dried milk in TBS. The membrane was then incubated with goat anti-p67-phox serum, and bound p67-phox was detected as described above for immunoblotting.

RESULTS

Results from several laboratories, including our own, support the hypothesis that phosphorylation at one or more serine residues in the C-terminus of p47-phox decreases the affinity of a C-terminal Arg/Lys domain (that encompasses the phosphorylation sites) for an internal binding site in the p47-phox tandem SH3 domains. Loss of affinity leads to relief of p47-phox self-association and unmasking of the SH3A domain binding site, which then engages the p22-phox PRR. The two p47-phox SH3 domains are linked by a short amino acid sequence that is rich in aspartic and glutamic acid residues. We speculated that the high-density negative charge of this linker peptide could bind the positively charged Arg/Lys domain in the p47-phox C-terminus. We predicted that lowering the net negative charge of this linker region would disrupt binding to the Arg/Lys domain of p47-phox and yield a constitutively active p47-phox. We generated full-length p47-phox mutants in which Asp-217 and Glu-218 (p47 D217A E218A) or Asp-217, Glu-218 and Glu-223 (p47 D217A E218A E223A) were replaced by alanines by site-directed mutagenesis, as described in the Materials and methods section (Figure 1).

The consequences of double and triple alanine substitutions for inter-SH3-domain linker binding were examined by the yeast two-hybrid binding method. Wild-type and alanine-substituted full-length p47-phox, and truncated p47-phox that encompasses the two SH3 domains plus the intervening linker sequence (p47 SH3AB), were expressed as fusion proteins with the B42 activation domain in EGY48 yeast. Yeast co-expressed the LexA binding domain fused to the p22-phox C-terminus, full-length p67-phox and the p47-phox Arg/Lys domain, and binding was detected as growth on leucine-deficient media. In contrast with wild-type p47 SH3AB, double-substituted p47 SH3AB D217A E218A and triple-substituted p47 SH3AB D217A E218A E223A did not bind the p47 Arg/Lys domain (Figure 3). A single alanine substitution had no effect on binding of p47 SH3AB to the Arg/Lys domain in the two-hybrid assay (results not shown). This indicates that loss of two or three of these negatively charged amino acids decreased the affinity of the p47-phox Arg/Lys domain for its target within the tandem SH3 domains. Full-length p47 D217A E218A E223A bound the p22-phox C-terminus, in contrast with wild-type p47-phox (Figure 3). This indicated that the SH3A domain was unmasked in p47 D217A E218A E223A, similar to p47 S310D S328D [22]. There was no difference in the binding of wild-type p47-phox and p47 D217A E218A E223A to p67-phox, which suggested that the introduced mutations did not affect p47-phox–p67-phox interactions.

Spontaneous binding of p47 D217A E218A E223A (Figure 3) and p47 S310D S328D [22] to p22-phox in the two-hybrid system suggested that these two substituted p47-phox proteins may be active in a cell-free NADPH oxidase system in the absence of stimulation by SDS or arachidonic acid. p47 S310D S328D was added to a semi-recombinant cell-free NADPH oxidase system consisting of neutrophil membranes and recombinant p67-phox and Rac1. Molar equivalent amounts of p47 S310D S328D and native p47-phox generated similar amounts of superoxide when arachidonate was used to activate NADPH oxidase activity (Figure 4, inset). Native p47-phox did not support superoxide generation in the absence of arachidonate stimulation (Figure 4).

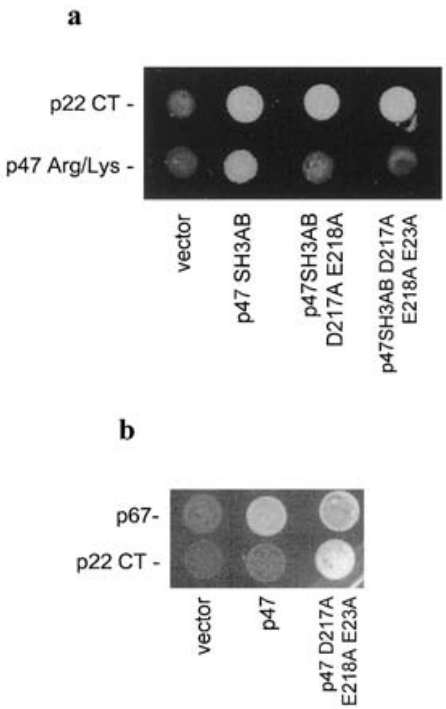

Figure 3 Effects of mutations in the inter-SH3 domain region of p47-phox on binding interactions

(**a**) Interactions between p47 SH3AB–B42 activation domain (ad) and p47 Arg/Lys domain–LexA binding domain (bd) fusion proteins in a yeast two-hybrid assay are shown. EGY48 yeast co-expressing p47 Arg/Lys fused to the LexA bd and B42 ad fused to (left to right) no insert, p47 SH3AB, p47 SH3AB D217A E218A and p47 SH3AB D217A E218A E223A were selected on galactose-containing histidine/uracil/tryptophan-deficient medium additionally lacking leucine (bottom row). Leucine prototrophy resulting from the interaction between p47 SH3AB and the p47 Arg/Lys domain was indicated as growth on medium (white spots). The D217A E218A and D217A E218A E223A mutants abrogated binding of p47 SH3AB to the p47 Arg/Lys domain. Mutations in the p47 SH3AB did not interfere with binding of the SH3A domain to the PRR in the p22-phox C-terminus (CT) (top row). (**b**) p47 D217A E218A E223A binds spontaneously to the p22-phox C-terminus. Yeast co-expressing the p22-phox C-terminus (CT) fused to the LexA bd, and the B42 ad fused to (left to right) no insert, full-length native p47-phox or full-length p47 D217A E218A E223A were selected on leucine-deficient medium. Leucine prototrophy resulting from binding of p47 D217A E218A E223A to the p22-phox C-terminus was indicated as growth on medium (white spots). Native p47-phox did not bind to the p22 C-terminus. The D217A E218A and E223A mutations in full-length p47-phox did not affect binding of the p47-phox C-terminal PRR to the p67-phox C-terminal SH3 domain (top row).

However, p47 S310D S328D was active without arachidonate, typically generating 20–25 % as much superoxide as did native p47-phox in the absence of arachidonate (Figure 4). Higher concentrations of p47 S310D S328D yielded proportionately increased NADPH oxidase activity (Figure 4).

p47-phox possesses an N-terminal PX domain. We reasoned that incorporating phosphatidylinositides into neutrophil membranes in the cell-free assay would increase overall NADPH oxidase activity by recruiting more p47-phox to the membrane. At 0.12 μM, p47 D217A E218A E223A supported little NADPH oxidase activity in neutrophil membranes that were not supplemented with PI-3-P (Figure 5). In contrast, p47 D217A E218A E223A incubated with 50 % PI-3-P-supplemented membranes, but without arachidonic acid, supported superoxide production similar to that obtained on incubation with arachidonic acid (Figure 5). p47 D217A E218A E223A was not as active as p47 S310D S328D on a molar equivalent basis (see Figure 7).

Unlike other PX domain-containing proteins that bind preferentially to PI-3-P, the PX domain of p47-phox has shown

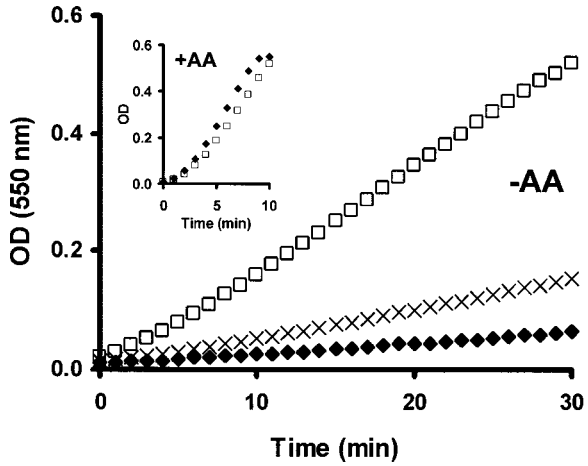

Figure 4 p47 S310D S328D is active in the cell-free system in the absence of arachidonic acid

Recombinant p67-phox (0.9 μM), Rac1 (0.2 μM) and p47 S310D S328D at concentrations of 0.12 μM (□) or 0.02 μM (×) or native p47-phox at 0.12 μM (◆) were incubated with deoxycholate-solubilized neutrophil membranes (10 μg/well) in the absence of arachidonic acid (− AA). The time course of superoxide generation (superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) was measured as described in the Materials and methods section. A comparative experiment with 0.12 μM native p47-phox (◆) or p47 S310D S328D (□) with arachidonic acid (30 μM) stimulation (+ AA) is shown in the inset. Results are representative of multiple similar experiments.

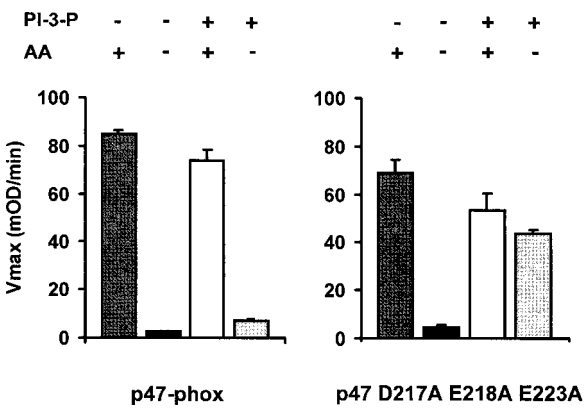

Figure 5 Enrichment of neutrophil membranes with PI-3-P enhances NADPH oxidase activity mediated by p47 D217A E218A E223A in the absence of arachidonic acid

Recombinant p67-phox (0.1 μM), Rac1 (0.2 μM) and 0.12 μM p47 D217A E218A E223A (right panel) or native p47-phox (left panel) were incubated with neutrophil membranes (10 μg/well) or 50% PI-3-P-supplemented membranes in the presence or absence of arachidonic acid (AA; 30 μM). Maximal rates of superoxide generation (V_{max}; superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) for each condition were measured as described in the Materials and methods section. Shown are results from two identical experiments.

higher affinity for PI-3,4-P in some [13], but not all [14], reports. Various proportions (weight of lipid/weight of neutrophil membrane protein) of PI-3-P and PI-3,4-P were co-solubilized with neutrophil membranes using deoxycholate to produce 0–50% PI-3-P/membrane and PI-3,4P/membrane mixtures (see the Materials and methods section). Mixtures of neutrophil membranes with PI-3-P or PI-3,4-P and p47 S310D S328D were incubated with p67-phox and Rac1 in the presence and absence of arachidonic acid (Figure 6). There were only marginal increases in superoxide production with increasing PI-3-P concentration

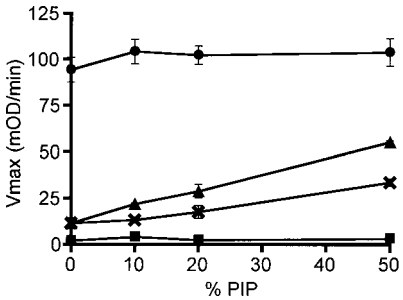

Figure 6 p47 S310D S328D-mediated NADPH oxidase activity in the cell-free system is enhanced in membranes supplemented with increasing percentages of PI-3-P and PI-3,4-P

Neutrophil membranes (10 μg/well) were enriched with 0%, 10%, 20% or 50% PI-3-P or PI-3,4-P as described in the Materials and methods. PI-3-P-enriched membranes were incubated with recombinant p67-phox (0.1 μM), Rac1 (0.2 μM), and p47 S310D S328D (0.12 μM) without (●) or with (▲) arachidonic acid (30 μM). PI-3,4-P-enriched membranes were similarly incubated with p47 S310D S328D without arachidonic acid (×). For comparison, native p47-phox (0.12 μM) was incubated under the same conditions in the absence of arachidonic acid (■). Maximal rates of superoxide generation (V_{max}; superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) for each condition were measured as described in the Material and methods section. Shown are means and ranges from two identical experiments, and the results are representative of multiple similar experiments. At 50% PI-3-P, NADPH oxidase activity with p47 S310D S328D in the absence of arachidonic acid was 74% of the activity seen with native p47-phox stimulated with arachidonic acid under identical conditions (results not shown).

when arachidonic acid was used to stimulate NADPH oxidase activity by p47 S310D S328D. In contrast, p47 S310D S328D-mediated NADPH oxidase activity was enhanced several-fold in membranes supplemented with 50% PI-3-P or PI-3,4-P compared with unsupplemented membranes (Figure 6). Native p47-phox supported no superoxide generation in the absence of arachidonate, irrespective of the concentration of PI-3-P (Figure 6) or PI-3,4-P (results not shown).

Enhancement by PI-3-P of the NADPH oxidase activity of p47 S310D S328D and p47 D217A E218A E223A was compared with the effects of other lipids. Neutrophil membranes were co-solubilized with PI-3-P, PI-4-P, PI or diOG and incubated with wild-type p47-phox, p47 S310D S328D or p47 D217A E218A E223A in the cell-free assay (Figure 7). Similar amounts of superoxide were generated in the presence of arachidonic acid stimulation for all lipids (Figure 7). In the absence of arachidonic acid, neutrophil membranes enriched in PI-3-P, PI-4-P or PI supported similar elevated levels of superoxide production by p47 S310D S328D and p47 D217A E218A E223A, in contrast with diOG, which did not augment oxidase activity over and above that in unsupplemented neutrophil membranes.

Additional experiments were performed using membranes enriched with negatively charged lipids (PG or PS) or the neutral lipid PC (Figure 8). In the absence of arachidonate, oxidase activity was enhanced for p47 S310D S328D with PG- and PS-supplemented membranes, but not following PC enrichment. Enhanced oxidase activity with PS-enriched membranes was similar to that with PI-3-P-enriched membranes, but we consistently saw greater superoxide production with PG-enriched membranes. p47 S310D S328D stimulated 14% more oxidase activity in PA- compared with PG-enriched membranes in the absence of arachidonic acid. Wild-type p47-phox did not support NADPH oxidase activity with any lipid-supplemented neutrophil membrane in the absence of arachidonate, except for membranes enriched with PG (Figure 8) or PA (results not shown), where small amounts of superoxide were seen. PG and PA appear to function as weak arachidonic acid mimetics [26] that are

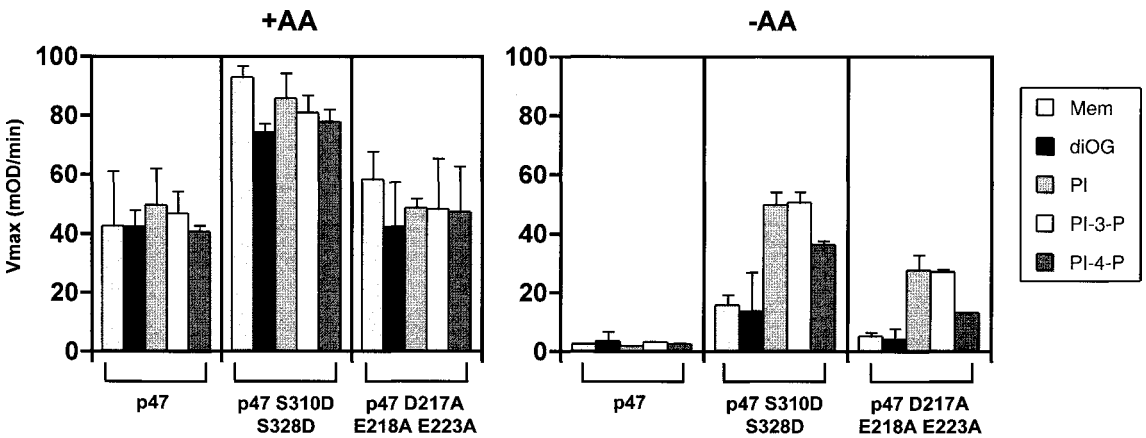

Figure 7 Phosphoinositide-dependence of p47 S310D S328D- and p47 D217A E218A E223A-mediated NADPH oxidase activity in the cell-free system

Recombinant p67-phox (0.1 μM), Rac1 (0.2 μM) and p47 S310D S328D (0.12 μM) or p47 D217A E218A E223A (0.12 μM) were incubated with neutrophil membranes (Mem; 10 μg/well) or membranes (10 μg/well) supplemented with 50 % diOG, PI, PI-3-P or PI-4-P in the absence (right panels) or presence (left panels) of arachidonic acid (AA; 30 μM). Maximal rates of superoxide generation (V_{max}; superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) for each condition were measured as described in the Materials and methods section. Shown are means and ranges for two identical experiments, and results are representative of several additional experiments.

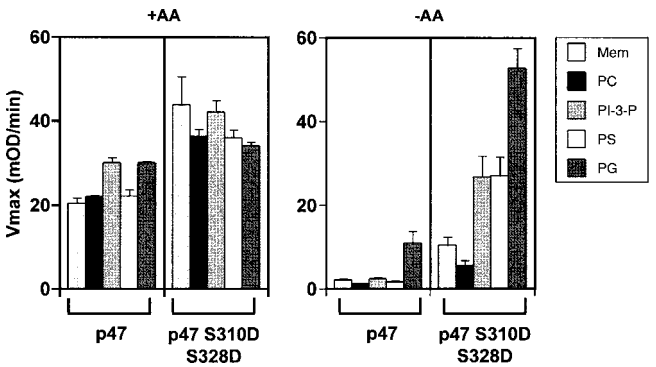

Figure 8 Effects of non-phosphoinositide lipids on p47 S310D S328D-mediated NADPH oxidase activity in the cell-free system

Recombinant p67-phox (0.1 μM), Rac1 (0.2 μM) and p47 S310D S328D (0.12 μM) or wild-type p47-phox (0.12 μM) were incubated with neutrophil membranes (Mem; 10 μg/well) or membranes (10 μg/well) supplemented with 50 % PC, PI-3-P, PS or PG in the absence (right panels) or presence (left panels) of arachidonic acid (AA; 30 μM). Maximal rates of superoxide generation (V_{max}; superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) for each condition were measured as described in the Materials and methods section. Shown are means and standard errors for two identical experiments with arachidonic acid and six identical experiments without arachidonic acid.

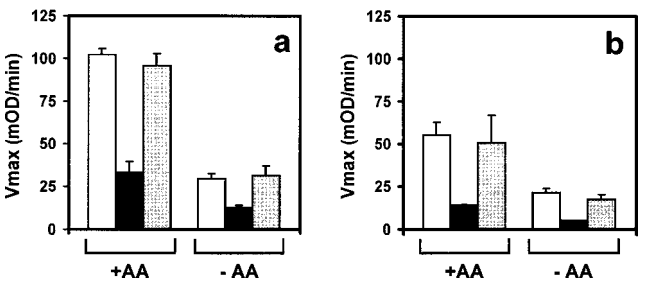

Figure 9 p47-phox Arg/Lys domain peptides inhibit NADPH oxidase activity mediated by p47 S310D S328D and p47 D217A E218A E223A

Recombinant p67-phox (0.1 μM), Rac1 (0.2 μM) and neutrophil membranes (10 μg/well) supplemented with 50 % PI-3-P were incubated with (**a**) p47 S310D S328D (0.12 μM) or (**b**) p47 D217A E218A E223A (0.12 μM) in the absence of arachidonic acid. p47-(306–335) (12.5 μM; solid bars), p47-(306–335) S310pS S328pS (12.5 μM; grey bars) or no peptide (white bars) was added to cell-free system incubation mixtures. Maximal rates of superoxide generation (V_{max}; superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) for each condition were measured as described in the Materials and methods section. Shown are means and ranges for two identical experiments, and results are representative of several other experiments.

capable of activating NADPH oxidase *in vitro* in the absence of arachidonic acid. Similar results were seen in experiments with p47 D217A E218A E223A (results not shown).

In an earlier paper, we showed that synthetic peptides that mimicked the p47-phox Arg/Lys domain encompassing Ser-310 and Ser-328 inhibited NADPH oxidase activity in the cell-free system [22]. A peptide with phosphoserines substituted for Ser-310 and Ser-328 was 100-fold less inhibitory. In the present study, we tested whether Arg/Lys domain peptides inhibited oxidase activity when incubated with full-length p47 S310D S328D or p47 D217A E218A E223A in the cell-free assay with and without arachidonate (Figure 9). At a concentration of 12.5 μM, p47-(306–335) profoundly inhibited both p47 S310D S328D- and p47 D217A E218A E223A-mediated NADPH oxidase activity, irrespective of the presence of arachidonic acid. Peptide p47-(306–335) S310pS S328pS was less inhibitory than the native sequence p47-(306–335). Higher concentrations of peptides inhibited proportionately oxidase activity mediated by both p47 S310D S328D and p47 D217A E218A E223A (results not shown), with the same concentration dependence as reported previously for native p47-phox in an arachidonic acid-stimulated cell-free system [22]. The fact that peptide p47-(306–335) inhibited oxidase activity mediated by p47 D217A E218A E228A as efficiently as that mediated by p47 S310D S328D means that the inter-SH3-domain linker sequence cannot be the sole binding target for p47-(306–335) and, by extension, for the corresponding sequence in the p47 Arg/Lys domain. In contrast with the p47 Arg/Lys domain peptides, inter-SH3-domain peptides were poor inhibitors of superoxide generation in the cell-free system. p47-(211–229) and p47-(211–229) D217A E218A E228A at 250 μM were incubated with p47 S310D S328D in the absence of arachidonic acid under the same experimental conditions as described in Figure 9. p47-(211–229) inhibited superoxide generation by 75 % (mean of two experiments),

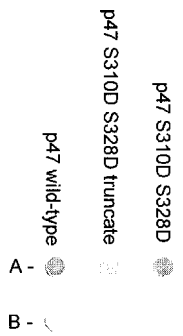

Figure 10 Recombinant truncated p47 S310D S328D binds poorly to p67-phox

Wild-type p47-phox, p47 S310D S328D truncate or p47 S310D S328D (20 pmol) (row A) or buffer control (row B) were spotted on a nitrocellulose membrane using a 96-well format dot-blot manifold (see the Materials and methods section). After blocking, 87 pmol of p67-phox was added to all wells and aspirated through the membrane with gentle vacuum. After blocking the entire membrane, p67-phox binding was detected as described in the Materials and methods section. The blot is representative of two identical experiments.

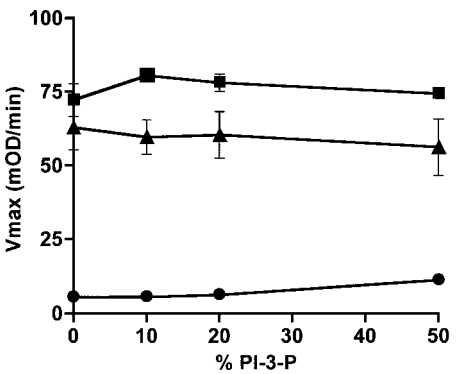

Figure 11 Truncated p47 310D S328D does not support cell-free NADPH oxidase activity in neutrophil membranes supplemented with PI-3-P in the absence of arachidonic acid

Recombinant p67-phox (0.1 μM), Rac1 (0.2 μM) and p47 S310D S328D truncate (0.12 μM) were incubated with neutrophil membranes (10 μg/well) or membranes supplemented with 10 %, 20 % or 50 % PI-3-P in the presence (▲) or absence (●) of arachidonic acid (30 μM). For comparison, native p47-phox (0.12 μM) was incubated under same conditions with arachidonic acid (■). Maximal rates of superoxide generation (V_{max}; superoxide dismutase-inhibitable reduction of ferricytochrome *c* recorded at 550 nm; OD = absorbance) for each condition were measured as described in the Materials and methods section. Shown are means and standard errors from three identical experiments.

whereas p47-(211–229) D217A E218A E228A resulted in only 45 % inhibition. Nearly identical degrees of inhibition by the two peptides were seen with p47 D217A E218A E228A in the cell-free system with and without arachidonic acid stimulation, and with wild-type p47-phox with arachidonic acid stimulation.

Arachidonic acid and SDS substitute for the physiological phosphorylation of p47-phox by unmasking the normally inaccessible N-terminal p47-phox SH3 domain binding site for the PRR of p22-phox [27]. N-terminal truncations of both p67-phox and p47-phox promote superoxide generation in the cell-free system, despite the absence of both p67-phox SH3 domains and the C-terminal p47-phox PRR respectively [28]. The ability to activate NADPH oxidase *in vitro* using p47 S310D S328D in the absence of arachidonic acid or SDS gave us an opportunity to re-examine the role of p67-phox SH3B–p47-phox C-terminal PRR binding in the cell-free system. On purification of p47 S310D S328D, GST–p47 S310D S328D and GFP–p47 S310D S328D, we saw evidence that p47 S310D S328D was cleaved at a unique sensitive site to yield a truncated p47 S310D S328D shortened by 5 kDa at the C-terminus (Figure 1). The missing sequence includes the PRR recognized by the p67-phox SH3 domain. As predicted, p67-phox bound native p47-phox and full-length p47 S310D S328D with greater avidity than truncated p47 S310D S328D in a blot binding assay (Figure 10). In the cell-free system, truncated p47 S310D S328D supported robust superoxide production on arachidonic acid stimulation (76 % of that by wild-type p47-phox using 50 % PI-3-P-supplemented membranes) (Figure 11). In contrast with full-length p47 S310D S328D (Figure 6), truncated p47 S310D S328D was poorly active in the cell-free system in the absence of arachidonic acid stimulation, even when using neutrophil membranes co-solubilized with high concentrations of PI-3-P (Figure 11). Taken together, these results suggested that SH3 domain-dependent p47-phox–p67-phox association is required for NAPDH oxidase activity in a cell-free system that is not stimulated by arachidonic acid or SDS.

DISCUSSION

Recombinant full-length p47-phox S310D S328D was spontaneously active in the *in vitro* NADPH oxidase system. This result is consistent with two previously published reports. Lopes et al. [29] showed that recombinant p47-phox phosphorylated *in vitro* by protein kinase C can reconstitute 25 % as much NADPH oxidase activity in the cell-free system in the absence of SDS compared with in its presence. Ago et al. [21] generated several recombinant full-length p47-phox proteins with mutations in the Arg/Lys domain that were active in the cell-free system in the absence of SDS stimulation. Taken together, these studies show that increasing negative charge at selected residues in the Arg/Lys domain, whether due to true phosphorylation [29], the introduction of negatively charged amino acids (Figure 4) or a decrease in positively charged amino acids [21], yielded the same result: reduced affinity of the Arg/Lys domain for the p47-phox tandem SH3 domains. Moreover, disrupting the Arg/Lys domain–p47 SH3AB association is sufficient to produce the activated p47-phox phenotype.

In a previous paper, we showed that the Arg/Lys region of p47-phox did not bind to either of the two individual SH3 domains in the yeast two-hybrid assay [22]. This suggested that the three-dimensional conformation of p47 SH3AB is important in defining the binding site for the Arg/Lys region. Using site-directed mutagenesis, we replaced glutamic acid and aspartic acid residues with alanines within the negatively charged, glutamic/aspartic acid-rich inter-SH3 domain linker region of both full-length p47-phox and p47 SH3AB. We hypothesized that loss of net negative charge in the linker region would weaken a putative electrostatic attraction between p47 SH3AB and the p47-phox Arg/Lys region and disrupt their internal association (effectively mimicking the p47-phox S310D S328D phenotype). Double and triple alanine substitutions did disrupt binding (Figure 3), suggesting a charge-dose effect whereby more than one negative charge needed to be neutralized.

However, association of the p47-phox Arg/Lys domain with p47 SH3AB cannot be explained wholly by this electrostatic interaction. The Arg/Lys domain peptide p47-(306–335) inhibited NADPH oxidase activity even when p47 D217A E218A E223A was used in the cell-free assay (Figure 8). If p47-(306–335) bound only to the inter-SH3 domain linker region, then p47 D217A E218A E223A should have been relatively resistant to inhibition compared with wild-type full-length p47-phox or p47

S310D S328D. The fact that p47 D217A E218A E223A was inhibitable even at low micromolar concentrations of Arg/Lys peptide suggests that amino acids 306–335 of p47-phox do not bind the inter-SH3 linker region directly. Another positively charged region within the p47-phox Arg/Lys domain may interact with the negatively charged linker site, leading to loss of the binding of p47 SH3AB D217A E218A E223A to the p47 Arg/Lys domain seen in the two-hybrid experiments (Figure 3). Alternatively, the D217A E218A E223A mutations may induce conformational changes that unfold p47 SH3AB and lead, in turn, to loss of a binding site distant from the inter-SH3 linker region for the p47 Arg/Lys domain. An internal interaction mediated by the linker region may stabilize the closed SH3AB conformation, and loss of this interaction due to the D217A E218A E223A substitutions may cause the mutated SH3AB structure to relax into the open, unmasked conformation. If true, this latter possibility suggests that the binding site for the p47 Arg/Lys domain is located elsewhere in p47 SH3AB, possibly comprising non-contiguous domains that are correctly spatially orientated only in properly folded p47 SH3AB. This view is also supported by the poor inhibition of superoxide generation by p47-(211–233) peptides, which is not consistent with existence of high-affinity binding of the negatively charged inter-SH3 region to another site within p47-phox.

p47-phox has a consensus PX domain in its N-terminus, and has been shown to bind to different PI-Ps, including PI-3,4-P and PI-3-P [13,14]. An interaction between p47-phox SH3B and an N-terminal PRR masks the PX domain and blocks spontaneous PI-P binding in quiescent neutrophils [12,13]. Conformational changes induced by phosphorylation in the Arg/Lys domain expose the PX domain and allow binding to phagosomal PI-Ps (analogous to unmasking of the SH3A domain and its subsequent association with p22-phox). The ability to perform the cell-free assay in the absence of arachidonic acid using spontaneously active full-length p47 S310D S328D or p47 D217A E218A E223A offered an opportunity to explore the effects of PI-Ps on NADPH oxidase activity *in vitro*. Neutrophil membranes enriched with every phosphatidylinositide tested showed markedly enhanced activity in the cell-free assay with both full-length p47 S310D S328D and p47 D217A E218A E223A in the absence of arachidonic acid (Figure 7). Enhancement was also seen with membranes supplemented with the negatively charged non-PI-P lipids PS, PG (Figure 8) and PA. In contrast, neutrophil membranes supplemented with the neutral lipids diOG and PC did not augment cell-free NADPH oxidase activity.

The PX domain of p47-phox uniquely possesses a second binding site that recognizes negatively charged non-PI-P phospholipids [13]. PA is the preferred non-PI-P phospholipid, with a 2.5-fold greater affinity than PS [13]. Supplementing PI-3,4-P-containing membranes with PA or PS synergistically increased their affinity for the p47-phox PX domain [13]. The presence of the second p47-phox PX domain site may explain the lack of specificity of PI-3,4P or PI-3P in increasing superoxide generation in the cell-free assay (Figure 6). The two p47-phox PX domain binding sites appear to have sufficiently similar affinities such that differences in PI-P compared with non-PI-P enhancement cannot be discerned under the experimental conditions used in the present studies. In contrast with p47-phox, p40-phox (which has a single PI-P binding site in its PX domain) specifically enhanced oxidase activity using neutrophil membranes supplemented with its preferred PI-3-P, but not with other PI-Ps or other negatively charged lipids (G. Peng and M. E. Kleinberg, unpublished work). This suggests that the cell-free system that we used is sensitive to PX-domain–PI-P interactions.

Our data show that NAPDH oxidase activity is enhanced in membranes supplemented with PI-P or negatively charged lipids compared with that in unsupplemented membranes. This may explain why oxidase activity was only partially reconstituted using *in vitro* phosphorylated p47-phox [29,30], truncated p47-phox [22,28] or full-length p47-phox with Arg/Lys domain mutations [21] in cell-free systems where supplemented membranes were not used. Negatively charged amphiphiles such as arachidonic acid may function as stimulators of NADPH oxidase activity by partitioning extensively into neutrophil membranes in the cell-free assay and mimicking negatively charged phospholipids. Arachidonic acid, therefore, has two effects *in vitro*: first, to induce conformational changes in p47-phox to unmask the SH3A and PX domains (mimicking phosphorylation), and secondly, to recruit p47-phox to membranes through PX domain binding. Using negatively charged lipid-supplemented neutrophil membranes and full-length p47 S310D S328D or p47 D217A E218A E223A, we were able to reconstitute consistently $> 70\,\%$, and often $100\,\%$, of NADPH oxidase activity *in vitro* compared with arachidonic acid-stimulated controls. It is not clear that additional putative effects of arachidonic acid on other phox proteins need to be invoked to account for incomplete reconstitution of oxidase activity in the cell-free assay with *in vitro* phosphorylated p47-phox [30].

Several important interactions between phox proteins have not been amenable to study in the arachidonic acid/SDS-activated cell-free system. One example is the interaction between the C-terminal SH3 domain of p67-phox and its target PRR in the p47-phox C-terminus. Studies in p67-phox-deficient Epstein–Barr virus B cells expressing p67-phox mutants lacking one or both SH3 domains showed that this interaction is critical for translocation from cytosol to membrane [31]. In contrast, truncated p47-phox lacking the C-terminal PRR and truncated p67-phox lacking both SH3 domains as well as the PB1 domain were both active in the cell-free system activated by arachidonic acid and SDS [28]. We used a truncated form of p47-phox S310D S328D to re-examine the role of the interaction between the p67-phox C-terminal SH3B domain and the p47-phox PRR in oxidase activation in our arachidonic acid-independent cell-free system. Truncated p47 S310D S328D supported superoxide generation to the same extent as did full-length p47 S310D S328D in the cell-free assay with arachidonic acid activation, but was inactive in the absence of arachidonic acid, in contrast with full-length p47 S310D S328D. This experiment confirmed *in vitro* that the interaction between the p67-phox SH3B domain and the p47-phox PRR is critical to the basic assembly of the active NADPH oxidase. This interaction may be important for recruitment of one of the phox proteins to cytochrome b_{558} or for the adoption of precise conformations of p47-phox and/or p67-phox within the oxidase complex. It is unclear how arachidonic acid and SDS co-opt this SH3 domain interaction in the anionic amphiphile-stimulated cell-free system.

In summary, spontaneously active mimics of phosphorylated p47-phox can be generated by mutations in the tandem SH3 domains and the C-terminal Arg/Lys phosphorylation domain of p47-phox. Supplementing neutrophil membranes with phosphatidylinositides and other negatively charged lipids increases the *in vitro* NADPH oxidase activity of p67-phox, Rac1 and spontaneously active p47-phox mutants to levels seen in the presence of arachidonic acid stimulation. It may be advantageous to avoid the use of arachidonic acid and SDS for stimulating NADPH oxidase activity under many cell-free conditions where phox protein interactions are under study. Improvements to the cell-free NADPH oxidase system described in the present paper may extend the use of this system in new studies.

This study was supported by National Institutes of Health grant AI40007.

REFERENCES

1 Babior, B. M. (1994) Activation of the respiratory burst oxidase. Environ. Health Perspect. **102**, 53–56
2 Nathan, C. and Shiloh, M. U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl. Acad. Sci. U.S.A. **97**, 8841–8848
3 Clark, R. A. (1999) Activation of the neutrophil respiratory burst oxidase. J. Infect. Dis. **179**, S309–S317
4 Babior, B. M., Lambeth, J. D. and Nauseef, W. (2002) The neutrophil NADPH oxidase. Arch. Biochem. Biophys. **397**, 342–344
5 Huang, J., Hitt, N. D. and Kleinberg, M. E. (1995) Stoichiometry of p22-*phox* and gp91-*phox* in phagocyte cytochrome *b*558. Biochemistry **34**, 16753–16757
6 Wallach, T. M. and Segal, A. W. (1996) Stoichiometry of the subunits of flavocytochrome *b*558 of the NADPH oxidase of phagocytes. Biochem. J. **320**, 33–38
7 Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosens, H. and Scrace, G. (1992) Cytochrome *b* -245 is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. Biochem. J. **284**, 781–788
8 Cross, A. R., Rae, J. and Curnutte, J. T. (1995) Cytochrome *b* -245 of the neutrophil superoxide-generating system contains two nonidentical hemes: potentiometric studies of a mutant form of gp91-*phox*. J. Biol. Chem. **270**, 17075–17077
9 Prigmore, E., Ahmed, S., Best, A., Kozma, R., Manser, E., Segal, A. W. and Lim, L. (1995) A 68-kDa kinase and NADPH oxidase component p67*phox* are targets for Cdc42Hs and Rac1 in neutrophils. J. Biol. Chem. **270**, 10717–10722
10 Dang, P. M.-C., Cross, A. R. and Babior, B. M. (2001) Assembly of the neutrophil respiratory burst oxidase: a direct interaction between p67*phox* and cytochrome *b*558. Proc. Natl. Acad. Sci. U.S.A. **98**, 3001–3005
11 Sumimoto, H., Hata, K., Mizuki, K., Ito, T., Kage, Y., Sakaki, Y., Fukumaki, Y., Nakamura, M. and Takeshige, K. (1996) Assembly and activation of the phagocyte NADPH oxidase: specific interaction of the N-terminal Src homology 3 domain of p47*phox* with p22*phox* is required for activation of the NADPH oxidase. J. Biol. Chem. **271**, 22152–22158
12 Hiroaki, H., Ago, T., Ito, T., Sumimoto, H. and Kohda, D. (2001) Solution structure of the PX domain, a target of the SH3 domain. Nat. Struct. Biol. **8**, 526–530
13 Karathanassis, D., Stahelin, R. V., Bravo, J., Perisic, O., Pacold, C. M., Cho, W. and Williams, R. L. (2002) Binding of the PX domain of $p47^{phox}$ to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. EMBO J. **21**, 5057–5068
14 Zhan, Y., Virbasius, J. V., Song, X., Pomerleau, D. P. and Zhou, G. W. (2002) *The* p40 *phox and* p47 *phox* PX domains of NADPH oxidase target cell membranes via direct and indirect recruitment by phosphoinositides. J. Biol. Chem. **277**, 4512–4518
15 Finan, P., Shimizu, Y., Gout, I., Hsuan, J., Truong, O., Butcher, C., Bennett, P., Waterfield, M. D. and Kellie, S. (1994) An SH3 domain and proline-rich sequence mediate an interaction between two components of the phagocyte NADPH oxidase complex. J. Biol. Chem. **269**, 13752–13755
16 Kuribayashi, F., Nunoi, H., Wakamatsu, K., Tsunawaki, S., Sato, K., Ito, T. and Sumimoto, H. (2002) The adaptor protein $p40^{phox}$ as a positive regulator of the superoxide-producing phagocyte oxidase. EMBO J. **21**, 6312–6320
17 Wientjes, F. B., Hsuan, J., Totty, N. F. and Segal, A. W. (1993) p40*phox*, a third cytosolic component of the activation complex of the NADPH oxidase to contain *src* homology 3 domains. Biochem. J. **296**, 557–561
18 El Benna, J., Faust, L. P. and Babior, B. M. (1994) The phosphorylation of the respiratory burst oxidase component p47*phox* during neutrophil activation: phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. J. Biol. Chem. **269**, 23431–23436
19 Rotrosen, D. and Leto, T. L. (1990) Phosphorylation of neutrophil p47-kDa cytosolic oxidase factor: translocation to membrane is associated with distinct phosphorylation events. J. Biol. Chem. **265**, 19910–19915
20 Fontayne, A., Dang, P. M.-C., Gougerot-Pocidalo, M.-A. and El Benna, J. (2002) Phosphorylation of p47*phox* sites by PKC α, βII, δ, and ζ: effect on binding to p22*phox* and on NADPH oxidase activation. Biochemistry **41**, 7743–7750
21 Ago, T., Nunoi, H., Ito, T. and Sumimoto, H. (1999) Mechanism for phosphorylation-induced activation of the phagocyte NADPH oxidase protein p47(phox). Triple replacement of serines 303, 304, and 328 with aspartates disrupts the SH3 domain-mediated intramolecular interaction in p47(phox), thereby activating the oxidase. J. Biol. Chem. **274**, 33644–33653
22 Huang, J. and Kleinberg, M. E. (1999) Activation of the phagocyte NADPH oxidase protein p47*phox*: phosphorylation controls SH3 domain-dependent binding to p22*phox*. J. Biol. Chem. **274**, 19731–19737
23 Golemis, E. A., Gyuris, J. and Brent, R. (1994) In Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R. , Kingston, R. E. , Moore, D. D. and Seigman, J. G., eds.), pp. 13.14.1–13.14.17, John Wiley and Sons, New York
24 Leto, T. L., Garrett, M. C., Fujii, H. and Nunoi, H. (1991) Characterization of neutrophil NADPH oxidase factors p47-*phox* and p67-*phox* from recombinant baculoviruses. J. Biol. Chem. **266**, 19812–19818
25 Kleinberg, M. E., Mital, D., Rotrosen, D. and Malech, H. L. (1992) Characterization of a phagocyte cytochrome *b*558 91-kilodalton subunit functional domain: identification of peptide sequence and amino acids essential for activity. Biochemistry **31**, 2686–2690
26 Palicz, A., Foubert, T. R., Jesaitis, A. J., Marodi, L. and McPhail, L. C. (2001) Phosphatidic acid and diacylglycerol directly activate NADPH oxidase by interacting with enzyme components. J. Biol. Chem. **276**, 3090–3097
27 Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minamaki, S. and Tageshige, K. (1994) Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase. Proc. Natl. Acad. Sci. U.S.A. **91**, 5345–5349
28 Hata, K., Ito, T., Takeshige, K. and Sumimoto, H. (1998) Anionic amphiphile-independent activation of the phagocyte NADPH oxidase in a cell-free system by p47*phox* and p67*phox*, both in C terminally truncated forms: implication for regulatory Src homology 3 domain-mediated interactions. J. Biol. Chem. **273**, 4232–4236
29 Lopes, L. R., Hoyal, C. R., Knaus, U. G. and Babior, B. M. (1999) Activation of the leukocyte NADPH oxidase by protein kinase C in a partially recombinant cell-free system. J. Biol. Chem **274**, 15533–15537
30 Shiose, A. and Sumimoto, H. (2000) Arachidonic acid and phosphorylation synergistically induce a conformational change of p47*phox* to activate the phagocyte NADPH oxidase. J. Biol. Chem. **275**, 13793–13801
31 de Mendez, I., Garrett, M. C., Adams, A. G. and Leto, T. L. (1994) Role of p67-*phox* SH3 domains in assembly of the NADPH oxidase system. J. Biol. Chem. **269**, 16326–16332

Received 18 October 2002/11 March 2003; accepted 21 March 2003
Published as BJ Immediate Publication 21 March 2003, DOI 10.1042/BJ20021629