Interactions between cytosolic components of the NADPH oxidase: p40^{phox} interacts with both p67^{phox} and p47^{phox}

Frans B. WIENTJES*‡, George PANAYOTOU†, Emer REEVES* and Anthony W. SEGAL*

*Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, and †Ludwig Institute for Cancer Research, 91 Riding House Street, London, U.K.

The NADPH oxidase of neutrophils and other bone-marrow-derived phagocytic cells is a multi-component system consisting of a flavocytochrome *b* in the plasma membrane and at least four cytosolic proteins. Three of the cytosolic proteins contain *src* homology 3 (SH3) domains, two each in p47^{phox} and p67^{phox}, and one in p40^{phox}. All three translocate from the cytosol to the flavocytochrome in the membrane upon stimulation of the cells. A small G-protein, p21^{rac}, is also involved in activation of the oxidase. The three cytosolic *phox* proteins occur as a complex in the cytosol and the strongest interaction appeared to be between p67^{phox} and p40^{phox}. We have investigated the interaction between p40^{phox} and the other two cytosolic *phox* proteins by *in vitro* binding assays. An affinity-bead approach was used as well as a biosensor technique (surface plasmon resonance). We observed the strongest attachment between p40^{phox} and p67^{phox} where the binding was between the N-terminal half of p67^{phox} and the C-terminal half of p40^{phox}, and did not appear to involve SH3 domains and proline-rich sequences. p40^{phox} also bound p47^{phox} but more weakly than it did p67^{phox}.

INTRODUCTION

Upon stimulation, neutrophils and other phagocytic cells undergo a respiratory burst during which superoxide is produced in the lumen of the phagocytic vacuole. The enzyme system responsible for superoxide production is the NADPH oxidase which consists of two transmembrane subunits, constituting a flavocytochrome *b* and a number of cytosolic components which play a role in the activation of the electron transport (for a recent review, see [1]).

Two cytosolic factors, p47^{phox} and p67^{phox}, were originally defined as being required for the oxidase [2,3] because they were found to be missing in patients with the autosomal recessive form of Chronic Granulomatous Disease (CGD), which is characterized by the absence of NADPH oxidase activity. A third cytosolic *phox* protein, p40^{phox}, was identified by its co-immunoprecipitation with p67^{phox} [4–6]. Its gene has been cloned and sequenced [4] and the derived amino acid sequence predicted homology with the N-terminal half of p47^{phox}. It is severely depleted in cells lacking p67^{phox} [4].

When the NADPH oxidase in neutrophils is stimulated by agonists like *N*-formyl-methionyl-leucyl-phenylalanine, or by the protein kinase C agonist phorbol myristate acetate (PMA), these three *phox* proteins translocate to the membrane [7] where they attach to the flavocytochrome *b* [4,8–11]. All three of these proteins contain *src*-homology 3 (SH3) domains, one in p40^{phox} [4] and two each in p47^{phox} and p67^{phox} [12–15]. SH3 domains are implicated in protein–protein interactions [16] and more specifically in directing signalling molecules to subcellular targets [17], and are thought to bind to proline-rich domains [18]. p47^{phox} and the small subunit of the flavocytochrome, p22^{phox}, both have several potential proline-rich motifs [19] and p67^{phox} has one (amino acids 227–234), providing numerous possibilities for interactions of the *phox* proteins with each other as well as with other proteins (e.g. cytoskeletal proteins, signalling proteins).

p21^{rac} was found to be necessary for activation of the NADPH oxidase in the cell-free assay [20,21] and there is evidence for involvement of p21^{rac} in the oxidase in the intact cell as well [22–24]. p21^{rac} translocates to the membrane on activation of the oxidase, but there is controversy as to which of the oxidase proteins it interacts with. *In vitro* experiments indicated an interaction with p67^{phox} [25] whereas other reports described an impaired translocation of p21^{rac} in p47^{phox}-deficient cells [26] and in cytochrome-deficient cells [27].

Interactions between the *phox* proteins have been explored by several groups using *in vitro* experiments. Binding of the SH3 domains of p47^{phox} to a proline-rich motif in the C-terminal part of p22^{phox} has been reported [19,28]. In one of these studies [28], evidence was also presented for the masking of the SH3 domains on p47^{phox} in the resting state by its own C-terminus. An interaction between p67^{phox} and the SH3 domains of p47^{phox} was also observed [28], which was inconsistent with other reports in which the interaction of these proteins was between a proline-rich motif in amino acids 358–371 on the C-terminal tail of p47^{phox} and the C-terminal SH3 domain of p67^{phox} [19,29,30].

Fuchs et al. [31] used the two-hybrid system to demonstrate interactions between p40^{phox} and both p47^{phox} and p67^{phox} with the SH3 domain of p40^{phox} interacting with p47^{phox} and the C-terminus of p40^{phox} attaching to p67^{phox}.

In this study we have investigated the binding between p40^{phox}, p47^{phox} and p67^{phox} in neutrophil cytosol, and with recombinant proteins on immobilized matrices and by plasmon resonance.

MATERIALS AND METHODS

Preparation of neutrophil cytosol

Neutrophils were prepared as described elsewhere [32] from buffy coat residues. PMA activation was for 3 min at 37 °C (10^8 cells/ml, 1 μg of PMA/ml) and the cells were broken by sonication in the presence of protease inhibitors as described previously [4,32]. The lysate was centrifuged at 350 ***g*** for 5 min at 4 °C and the supernatant was then centrifuged in a Beckman TLX ultracentrifuge at 125000 ***g*** at 4 °C for 15 min (TLA 100.4 rotor). The supernatant of this run provided the neutrophil cytosol.

Abbreviations used: SH3, *src* homology 3; GST, glutathione S-transferase; PMA, phorbol myristate acetate.

‡ To whom correspondence should be addressed.

Immunoaffinity chromatography and Western blotting

Affinity-purified anti-p67^{phox} antibodies (0.4 mg of protein) were coupled to a 1-ml Hitrap column (Pharmacia) as described previously [4]. An aliquot (1 ml) of cytosol was passed through the column twice to remove p67^{phox} and the cytosol was assessed for the presence of the cytosolic *phox* proteins by Western blotting.

A sample (20 μg) of protein was run on 12.5 % polyacrylamide gels and the proteins were transferred on to a nitrocellulose filter electrophoretically. The antibodies used to detect the *phox* proteins on the immunoblots were raised in rabbits against recombinant proteins made with the pGEX expression system (Pharmacia) [33]. The antibodies were affinity-purified as described previously [4] and the enhanced chemiluminescence (ECL) system (Amersham) was used to develop the blots.

Recombinant proteins

Full-length cDNAs coding for p67^{phox} and p47^{phox} were cloned into the *Bam*HI–*Eco*RI site of pGEX.2T (Pharmacia) in-frame with the glutathione S-transferase (GST) gene as described [33]. Because the p40^{phox} cDNA contained an internal *Bam*HI site, as did the p47^{phox} and p67^{phox} cDNAs, cloning of the p40^{phox} cDNA was done in a similar way to that described for p47^{phox} and p67^{phox}: using a unique *Nco*I site at the beginning of the p40^{phox} coding sequence, the p40^{phox} cDNA was cloned into the pGEX vector into which the first 130 nucleotides of the p40^{phox} coding sequence had been cloned by PCR.

The N-terminal truncated proteins were made by choosing an appropriate restriction site together with *Eco*RI to cut out a 3′ part of the gene, filling in the remaining plasmid with Klenow DNA polymerase to blunt end and religating. Mutant genes deleted in SH3 domains were created using the pAlter system (Promega). Other constructs were made by PCR using primers containing the appropriate restriction sites. In all cases, the correct sequence was confirmed by nucleotide sequencing using an ABI automated sequencer. Figure 1 shows the different constructs made.

Plasmids were transformed into *Escherichia coli* XL1Blue and proteins were induced with isopropylthiogalactoside (0.1 mM) at 37 °C or at lower temperatures (20–26 °C) where the proteins were insoluble. After harvest, the bacteria were washed twice with 50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA containing protease inhibitors (1 mM di-isopropylfluorophosphate, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 μg/ml tosyl-lysylchloromethane). The bacteria were lysed by one cycle of freezing/thawing followed by sonication (3 × 30 s, on ice). After removing the cell debris by centrifugation, the fusion proteins were purified using glutathione–Sepharose (Pharmacia) and cut with thrombin while bound on the glutathione beads [10–20 units of thrombin (Sigma) per ml for 10 min at room temperature]. The final purification was by ion-exchange chromatography. All proteins were checked for purity and stability by SDS/PAGE.

Binding assay on beads

Samples (2–5 mg) of the recombinant full-length or mutant *phox* proteins were bound covalently to 1-ml Hitrap columns, and the efficiency of binding was determined by measuring the protein concentration before and after coupling with a protein dye method (Bio-Rad). The loaded beads were extracted from the columns and used in a batch procedure. They were blocked with Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.05 % Tween 20, 1 % (w/v) gelatin and 3 % (w/v) BSA.

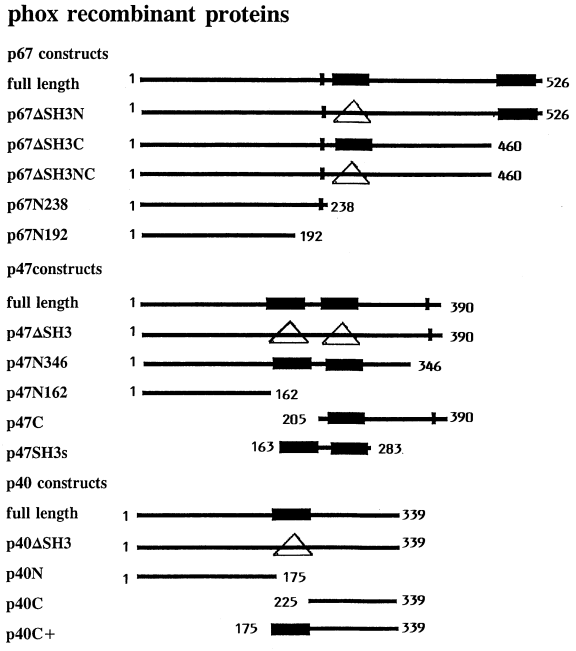

Figure 1 Schematic representation of the *phox* protein constructs used in this study

All genes were cloned at the 5′ end in frame after the GST gene in pGEX.2T. The proteins are presented as bars with the positions of the SH3 domains and deletions (△) indicated. The numbers left and right of the bars are first and last amino acids, respectively.

For binding, usually 200 pmol of a full-length *phox* protein was incubated with an equimolar amount of a series of truncated/deleted variants of another *phox* protein bound to beads. The same volume of beads was used in all samples. The negative control consisted of de-activated beads without protein. Incubation was for 15 min at room temperature with constant agitation. The beads were washed three times with 20 vol. of wash buffer (Tris-buffered saline plus 0.05 % Tween 20), resuspended in sample buffer and subjected to SDS/PAGE and Western blotting. The *phox* protein binding to the beads was determined with rabbit antibodies (see above) and ^{125}I-Protein A (Amersham) and quantified with a Fujibas phosphorimager.

Strongest binding was usually observed with the full-length *phox* protein bound to beads, and binding to mutated forms is expressed as a percentage of that.

Surface plasmon resonance with the BIAcore instrument

The basic principles of operation of the Pharmacia instrument BIAcore have been described by Jonsson et al. [34]. The running buffer consisted of 20 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005 % Tween-20, and proteins were transferred to this buffer using small disposable desalting columns (Pharmacia). For the measurement of p40^{phox}/p67^{phox} interactions, a goat antiserum against GST, supplied by Pharmacia, was immobilized on a biosensor surface activated by *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pharmacia) for coupling through free amino groups. Unreacted groups were blocked with an injection of 1 M ethanolamine and non-covalently associated material was removed by two 4-μl pulses of 0.2 M glycine, pH 2.2, 0.05 % SDS. Purified GST–p40^{phox} was injected over the immobilized antibody

at a concentration of 100 μg/ml followed by running buffer for 5 min after which time a stable baseline was attained. Purified p67^{phox} protein was then injected at various concentrations and its interaction with the antibody-bound GST–p40^{phox} monitored. The response obtained upon injection of p67^{phox} on antibody alone (no GST–p40^{phox}) was subtracted to correct for small differences in the bulk refractive index, due to non-associating protein and buffer differences. The association and dissociation kinetics were analysed with the BIA evaluation software supplied with the instrument, as described [35].

RESULTS

Immunoabsorption of p40^{phox} together with p67^{phox}

In an initial experiment we investigated whether all p40^{phox} and p47^{phox} are in a complexed form with p67^{phox}. Cytosol from resting cells (1 ml containing approx. 10 mg of protein) was passed through a column containing antibodies to p67^{phox}. Western blotting showed that all of both p67^{phox} and p40^{phox} were extracted from the cytosol by this column (Figure 2). Also most, but not all, of the p47^{phox} was extracted (results not shown).

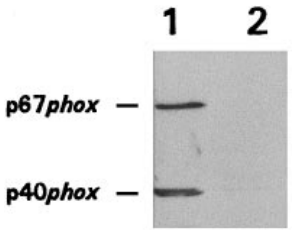

Figure 2 Absorption of p40^{phox} on an anti-p67^{phox} affinity column

Immunoblot of neutrophil cytosol (prepared from resting cells) before (1) and after (2) passage through an anti-p67^{phox} affinity column. The blot was stained for p40^{phox} and p67^{phox}. The bands were visualized with the ECL system using goat anti-(rabbit IgG) horseradish peroxidase-conjugated second antibodies.

Table 1 Binding of recombinant p40^{phox} to p67^{phox} and p47^{phox} and vice versa

200 pmol of protein bound to beads was used (either full-length or mutated protein as indicated). Incubation was with 200 pmol of the complementary *phox* protein and after SDS/PAGE and Western blotting, the amount of protein was determined with ^{125}I-Protein A and a phosphorimager. Values are given as percentages (with binding to full-length protein taken as 100%) and are the means $\pm$ S.D. of 4–6 determinations.

Protein on beads	Relative p40 binding (%)	Relative p67 binding (%)	Relative p47 binding (%)
p67^{phox}	100	–	–
p67N192	59 $\pm$ 22	–	–
p67N238	51 $\pm$ 20	–	–
p67ΔSH3C	74 $\pm$ 16	–	–
p67ΔSH3N	68 $\pm$ 20	–	–
p47^{phox}	100	–	–
p47N346	0	–	–
p47SH3s	0	–	–
p47ΔSH3	0	–	–
p47C	49 $\pm$ 11	–	–
p40^{phox}	–	100	100
p40ΔSH3	–	39 $\pm$ 16	39 $\pm$ 13
p40N	–	5 $\pm$ 5	10 $\pm$ 9
p40C	–	45 $\pm$ 17	17 $\pm$ 7
p40C+	–	100	100

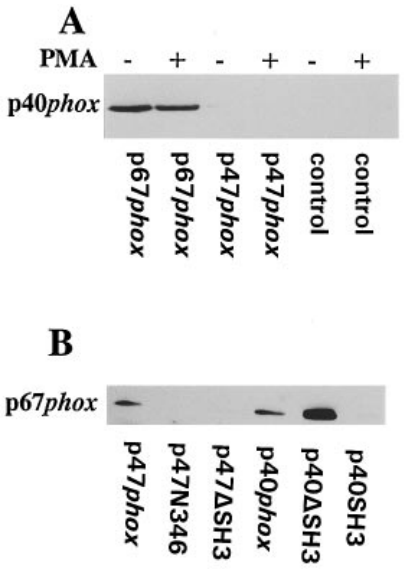

Figure 3 Binding of *phox* proteins from cytosol to affinity beads

Beads (200 pmol of protein) were incubated with cytosol (0.3 ml; 3 mg of protein) and were processed as described in the Materials and methods section and, after immunostaining, bands were visualized with the ECL system. (**A**) p40^{phox} pulled out of cytosol by p67^{phox} on beads but not by p47^{phox}. Cytosol was from resting (lanes 1, 3 and 5; lanes numbered from left to right) or PMA-activated cells (lanes 2, 4 and 6). Lanes 1 and 2: p67^{phox} beads; lanes 3 and 4: p47^{phox} beads; lanes 5 and 6: control beads. Blot was stained for p40^{phox}. (**B**) p67^{phox} pulled out of cytosol from resting neutrophils by p47^{phox} and p40^{phox} on beads. Beads contained: 1, p47^{phox}; 2, p47N346; 3, p47ΔSH3; 4, p40^{phox}; 5, p40ΔSH3; 6, p40SH3. Blot was stained for p67^{phox}.

Binding to full-length and modified recombinant proteins on beads

Binding of p40^{phox} to p67^{phox} and p47^{phox} constructs

p40^{phox} bound to full-length p67^{phox} (Table 1). It also bound equally to constructs of the first 192 and 238 amino acids, indicating that the binding site for p40^{phox} is towards the N-terminus of p67^{phox} and does not involve the proline-rich motif in position 226–235.

p40^{phox} also bound to p47^{phox} although with lower affinity. Binding of p40^{phox} to p47^{phox} beads was approx. 20% of that to p67^{phox} beads in molar terms (22 $\pm$ 8%, $n = 5$). Binding of p40^{phox} was not observed to truncated forms of p47^{phox} consisting of the N-terminal 162 (results not shown) or 346 amino acids, p47^{phox} deleted in both SH3 domains, or to the SH3 domains themselves (Table 1). Some binding was observed to the C-terminal half of p47^{phox}.

When cytosol from resting or PMA-activated cells was passed over the beads, p40^{phox} from both activated and non-activated cytosol was found to bind to p67^{phox}, whereas no p40^{phox} bound to the p47^{phox} beads or control beads (Figure 3A). Since p40^{phox} and p67^{phox} are already complexed in the cytosol, this presumably means that the interaction between the two proteins is reversible, allowing p40^{phox} to interact with p67^{phox} on the beads. On the other hand, the interaction between p40^{phox} and p47^{phox} is apparently too weak for a detectable binding to occur under these conditions (see also the section on the biosensor experiments).

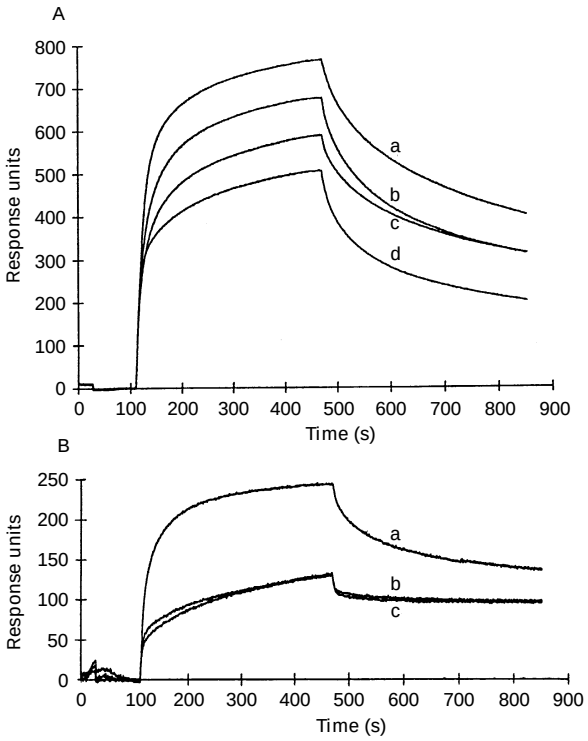

Figure 4 Measurement of binding of full-length and mutant p67^{phox} to p40^{phox} by plasmon resonance

GST–p40^{phox} fusion protein was immobilized on anti-GST antibody on a sensor chip and the p67^{phox} variants were injected at $t = 100$ s. Injection was ended at $t = 480$ s. Binding is recorded in arbitrary response units. (**A**) p67^{phox} and its SH3 deletions. Curve a, p67ΔSH3C; b, full-length p67^{phox}; c, p67ΔSH3N; d, p67ΔSH3NC. (**B**) p67^{phox} and its truncated forms. Curve a, full-length p67^{phox}, b, p67N192; c, p67N238. Different protein concentrations were used in the experiments of (**A**) and (**B**).

Binding of p67^{phox} and p47^{phox} to p40^{phox} constructs

Table 1 also shows the binding of p47^{phox} and p67^{phox} to constructs of p40^{phox}. Binding to full-length p40^{phox} is shown as well as to the protein with its SH3 domain deleted and to constructs consisting of the N-terminal half, ending before the SH3 domain, and of the C-terminal half, with and without the SH3 domain.

Both p67^{phox} and p47^{phox} bound to full-length p40^{phox} on beads. It was not possible to make direct comparisons of the relative quantities of each protein bound because of the different binding efficiencies of the two antisera used for quantification.

Little binding of either protein was observed to the N-terminal half of p40^{phox}. Both proteins bound to the C-terminus containing the SH3 domain, and this interaction was reduced, in the case of p47^{phox} almost abolished, by removal of the SH3 domain. Binding of both proteins was also reduced to about half by the removal of the SH3 domain from the full-length p40^{phox}. This suggests a role for the p40^{phox} SH3 domain in binding p47^{phox}, although this binding was not dependent upon the SH3 domain alone because there was some binding of p47^{phox} to p40ΔSH3. The p40^{phox} SH3 domain alone, or as a GST fusion protein, gave inconsistent results (not shown) indicative of weak binding.

With respect to p67^{phox}, its binding to p40C is consistent with its binding to p40ΔSH3 which was also observed when cytosol was incubated with the beads: both p40^{phox} and p40ΔSH3 bound p67^{phox} from neutrophil cytosol (Figure 3B).

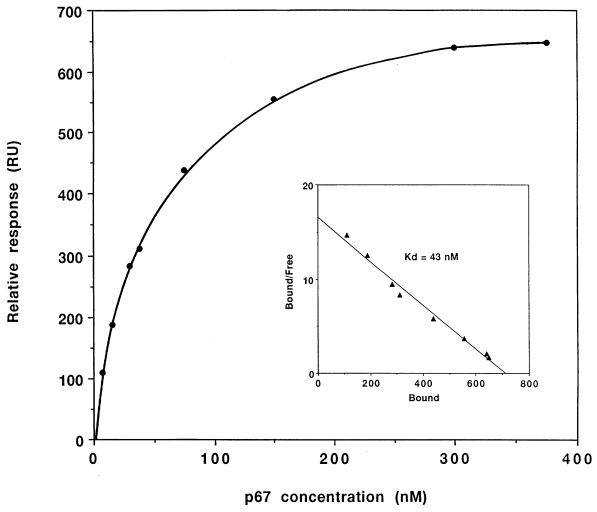

Figure 5 Binding constant of binding of p67^{phox} to immobilized GST–p40^{phox}

A series of concentrations of p67^{phox} were injected over immobilized GST–p40^{phox} and the response obtained at equilibrium was plotted. The insert shows the transformation of the data to a Scatchard plot, the slope of which was used to derive the binding constant of the interaction.

Interaction between p47^{phox} and p67^{phox}

When cytosol from resting or PMA-activated cells was passed over beads containing immobilized recombinant p47^{phox} or p67^{phox}, binding of the complementary protein was observed. Figure 3(B) shows binding of cytosolic p67^{phox} to p47^{phox} beads.

Binding of the recombinant full-length and mutant proteins to the full-length complementary protein on the beads was also observed but was not very strong. The data (not shown) indicated that p47^{phox} interacted with p67^{phox} and that the C-terminal SH3 domain of p67^{phox} was involved in this interaction.

p67^{phox} bound to p47^{phox} but not to p47N346, to the SH3 domains and only sparingly to the protein deleted in the SH3 domains. This could be interpreted as an (apparently weak) interaction between the C-terminal SH3 domain of p67^{phox} and the C-terminus of p47^{phox}.

Surface plasmon resonance measurements

In order to corroborate these findings and to obtain quantitative data on the kinetics and affinities involved we investigated the interactions of these proteins by surface plasmon resonance with the BIAcore instrument.

Purified GST–p40^{phox} was immobilized on an anti-GST antibody on a sensor chip and the various p67^{phox} mutant proteins were injected. The data in Figure 4(A) show a comparison between full-length p67^{phox} and its SH3-deleted mutants. Figure 4(B) compares full-length p67^{phox} with p67N192 and p67N238, the N-terminal region of p67^{phox} without and with the proline-rich motif, respectively. All proteins bound to p40^{phox} in agreement with the results obtained with the affinity beads.

In order to estimate the affinity of the p40^{phox}/p67^{phox} interaction, various concentrations of p67^{phox} were injected over the immobilized p40^{phox}. The response at equilibrium (in arbitrary resonance units) was plotted versus the p67^{phox} concentration, and the data analysed in a Scatchard plot (Figure 5). This analysis gave a binding constant of 43 nM, indicative of a high-affinity interaction. The association and dissociation rate constants were also estimated directly from the interaction curves

(results not shown). Only at low concentrations of $p67^{phox}$ did the observed responses fit into a simple, one-to-one binding model, with an association rate constant of $4.9 \times 10^5\ M^{-1} \cdot s^{-1}$ and a dissociation rate constant of $5 \times 10^{-3}\ s^{-1}$. These numbers should only be considered as approximate estimates of the kinetic constants.

Binding of $p47^{phox}$ to $p40^{phox}$ was also observed in the BIAcore system, but the results were not consistent, presumably due to the lower affinity between these proteins.

DISCUSSION

This study has revealed that the strongest interaction of the *phox* proteins is between $p67^{phox}$ and $p40^{phox}$, and that all the $p40^{phox}$ in cytosol is bound to $p67^{phox}$. This binding occurs between the N-terminus of $p67^{phox}$ and the C-terminus of $p40^{phox}$. Neither the proline-rich domain nor the SH3 domains of $p67^{phox}$ are required, whereas the SH3 domain of $p40^{phox}$ enhances the association. $p21^{rac}$ also binds to $p67^{phox}$ in its N-terminal domain [25]. We did not observe competition between $p40^{phox}$ and $p21^{rac}$ for binding to $p67^{phox}$ (results not shown).

$p40^{phox}$ binds less strongly to $p47^{phox}$ than to $p67^{phox}$. Binding to $p47^{phox}$ occurs between the C-terminus of $p47^{phox}$, which contains a proline-rich domain and an SH3 domain, and the C-terminus and SH3 domain of $p40^{phox}$. This result is less easy to interpret because if $p40^{phox}$ binds to the C-terminal part of $p47^{phox}$, it would also be expected to bind to the $p47^{phox}$ construct deleted in the SH3 domains, which it does not. It is possible that the construct lacking the SH3 domains has additional conformational changes that further compromise the already weak affinity of $p40^{phox}$ for $p47^{phox}$. The binding domain for the SH3 domain of $p40^{phox}$ in the C-terminal part of $p47^{phox}$ could involve one of the three proline-rich sequences located in this region [19]. Figure 6 is a model which summarizes the interactions between the *phox* proteins.

One previous study examined the association between these proteins using the two-hybrid system [31]. This system only detects the presence or absence of an interaction rather than the relative strength of such an interaction. It was observed that the C-terminus of $p40^{phox}$ was required for interaction with $p67^{phox}$, without requirement of the SH3 domain for binding to $p67^{phox}$. These authors also found that the $p40^{phox}$ SH3 domain bound to $p47^{phox}$. That study did not investigate the regions of $p67^{phox}$ and $p47^{phox}$ required for binding to $p40^{phox}$.

Four studies [19,28–30] have investigated the interactions between $p47^{phox}$ and $p67^{phox}$, which we found to be much weaker than those involving $p40^{phox}$ and $p67^{phox}$ or $p47^{phox}$. Three of these studies had similar findings to those in the current study, with a dominant interaction between the C-terminal SH3 domain of $p67^{phox}$ and the C-terminus of $p47^{phox}$.

The description of the associations between $p40^{phox}$ and $p67^{phox}$ and $p47^{phox}$ provide for a wide variety of interactions between the *phox* proteins. These proteins also bind to the cytoskeleton and the flavocytochrome *b* in the membrane, as well as to the small G-protein $p21^{rac}$. Detailed understanding of these interactions and their variation with activation will help our understanding of the mechanism by which electron transport is accomplished across the wall of the phagocytic vacuole.

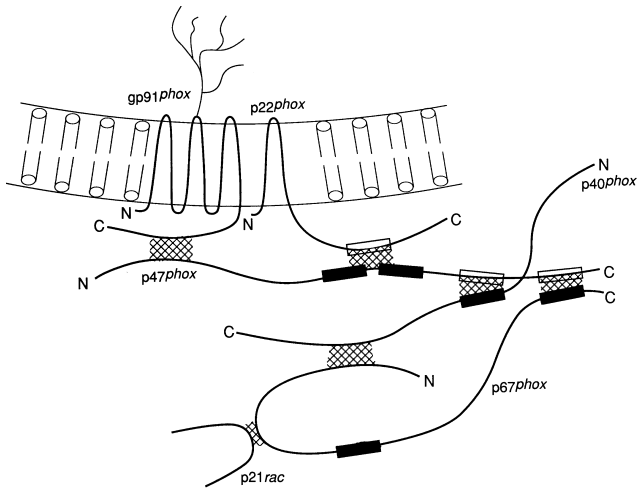

Figure 6 Model showing the interactions between the *phox* proteins

This model shows the interactions between the NADPH oxidase components as found by us and others. Black boxes are SH3 domains, open boxes the proline-rich sequences interacting with them.

We thank Angela Boyhan and Carmel Teahan for making some of the plasmids, Michael Haas for assistance in purification of proteins and Keith Parker for help with DNA sequencing. The work described here was supported by the Wellcome Trust.

REFERENCES

1 Thrasher, A. J., Keep, N. H., Wientjes, F. and Segal, A. W. (1994) Biochim. Biophys. Acta **1227**, 1–24
2 Segal, A. W., Heyworth, P. G., Cockcroft, S. and Barrowman, M. M. (1985) Nature (London) **316**, 547–549
3 Volpp, B. D., Nauseef, W. M. and Clark, R. A. (1988) Science **242**, 1295–1297
4 Wientjes, F. B., Hsuan, J. J., Totty, N. F. and Segal, A. W. (1993) Biochem. J. **296**, 557–562
5 Someya, A., Nagaoka, I. and Yamashita, T. (1993) FEBS Lett. **330**, 215–218
6 Mizunari, H., Karatsuji, T., Namiki, H. and Tsunawaki, S. (1994) Biochem. Biophys. Res. Commun. **198**, 191–199
7 Clark, R. A., Volpp, B. D., Leidal, K. G. and Nauseef, W. M. (1990) J. Clin. Invest. **85**, 714–721
8 Heyworth, P. G., Shrimpton, C. F. and Segal, A. W. (1989) Biochem. J. **260**, 243–248
9 Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H. and Clark, R. A. (1991) J. Clin. Invest. **87**, 352–356
10 Rotrosen, D., Kleinberg, M. E., Nunoi, H., Leto, T. L., Gallin, J. I. and Malech, H. L. (1990) J. Biol. Chem. **265**, 8745–8750
11 Nakanishi, A., Imajoh-Ohmi, S., Fujinawa, T., Kikuchi, H. and Kanegasaki, S. (1992) J. Biol. Chem. **267**, 17072–17074
12 Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I. and Malech, H. L. (1989) Science **245**, 409–412
13 Volpp, B. D., Nauseef, W. M., Donelson, J. E., Moser, D. R. and Clark, R. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7195–7199
14 Rodaway, A. R., Teahan, C. G., Casimir, C. M., Segal, A. W. and Bentley, D. L. (1990) Mol. Cell. Biol. **10**, 5388–5396
15 Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M. G., Nauseef, W. M., Clark, R. A., Gallin, J. I. and Malech, H. L. (1990) Science **248**, 727–730
16 Musacchio, A., Gibson, T., Lehto, V. and Saraste, M. (1992) FEBS Lett. **307**, 55–61
17 Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. and Schlessinger, J. (1993) Cell **74**, 83–91
18 Ren, R., Mayer, B. J., Cichetti, P. and Baltimore, D. (1993) Science **259**, 1157–1161
19 Leto, T. L., Adams, A. G. and de Mendez, I. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 10650–10654
20 Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G. and Segal, A. W. (1991) Nature (London) **353**, 668–670
21 Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T. and Bokoch, G. M. (1991) Science **254**, 1512–1515
22 Dorseuil, O., Vazquez, A., Lang, P., Bertoglio, J., Gacon, G. and Gerald, L. (1992) J. Biol. Chem. **267**, 20540–20542
23 Voncken, J. W., Van Schaick, H., Kaartinen, V., Deemer, K., Coates, T., Landing, B., Pattengale, P., Dorseuil, O., Bokoch, G. M., Groffen, J. and Heisterkamp, N. (1995) Cell **80**, 719–728
24 Gabig, T. G., Crean, C. D., Mantel, P. L. and Rosli, R. (1995) Blood **85**, 804–811
25 Diekmann, D., Abo, A., Johnston, C., Segal, A. W. and Hall, A. (1994) Science **265**, 531–533

26 El Benna, J., Ruedi, J. M. and Babior, B. M. (1994) J. Biol. Chem. **269**, 6729–6734
27 Heyworth, P. G., Bohl, B. P., Bokoch, G. M. and Curnutte, J. T. (1994) J. Biol. Chem. **269**, 30749–30752
28 Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minakami, S. and Takeshige, K. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 5345–5349
29 Finan, P., Shimizu, Y., Gout, I., Hsuan, J., Truong, O., Butcher, C., Bennett, P., Waterfield, M. D. and Kellie, S. (1994) J. Biol. Chem. **269**, 13752–13755
30 Leusen, J. H., Fluiter, K., Hilarius, P. M., Roos, D., Verhoeven, A. J. and Bolscher, B. G. (1995) J. Biol. Chem. **270**, 11216–11221
31 Fuchs, A., Dagher, M. C. and Vignais, P. V. (1995) J. Biol. Chem. **270**, 5695–5697
32 Segal, A. W. and Jones, O. T. G. (1980) FEBS Lett. **110**, 111–114
33 Abo, A., Boyhan, A., West, I., Thrasher, A. J. and Segal, A. W. (1992) J. Biol. Chem. **267**, 16767–16770
34 Jonsson, U., Fagerstam, L., Roos, H., Ronnberg, J., Sjolander, S., Stenberg, E., Stahlberg, R., Urbaniczky, C., Ostlin, H. and Malmqvist, M. (1991) Biotechniques **11**, 520–527
35 Panayotou, G., Gish, G., End, P., Truong, O., Gout, I., Dhand, R., Fry, M. J., Hiles, I., Pawson, T. and Waterfield, M. D. (1993) Mol. Cell. Biol. **13**, 3567–3576

Received 25 January 1996/2 April 1996; accepted 18 April 1996