p40^{phox} participates in the activation of NADPH oxidase by increasing the affinity of p47^{phox} for flavocytochrome b_{558}

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NADPH oxidase is one of the major components of the innate immune system and is used by phagocytes to generate microbicidal reactive oxygen species. Activation of the enzyme requires the participation of a minimum of five proteins, p22^{phox}, gp91^{phox} (together forming flavocytochrome b_{558}), p47^{phox}, p67^{phox} and the GTP-binding protein, Rac2. A sixth protein, p40^{phox}, has been implicated in the control of the activity of NADPH oxidase principally based on its sequence homology to, and physical association with, other *phox* components, and also the observation that it is phosphorylated during neutrophil activation. However, to date its role in regulating the activity of the enzyme has remained obscure, with evidence for both positive and negative influences on oxidase activity having being reported. Data are presented here using the cell-free system for NADPH oxidase activation that shows that p40^{phox} can function to promote oxidase activation by increasing the affinity of p47^{phox} for the enzyme approx. 3-fold.

Key words: host defence, oxygen radicals, p47^{phox}, respiratory burst, superoxide.

INTRODUCTION

The NADPH oxidase of phagocytic cells plays a pivotal role in innate immunity. When activated, the function of the enzyme is to generate microbicidal reactive oxygen species derived from the primary product of NADPH oxidase, superoxide (O_2^-) (reviewed by Nauseef [1]). The active oxidase requires the participation of five gene products; two of these, p22^{phox} and gp91^{phox}, together form flavocytochrome b_{558}, the membrane-bound core of the enzyme. gp91^{phox} contains all the catalytic machinery necessary to oxidize NADPH and form O_2^-; the NADPH-binding site, one molecule of FAD and two molecules of haem ([2,3] and L. Yu, M. C. Dinauer and A. R. Cross, unpublished work). In order to convert the normally inactive oxidase into the active state, three other proteins are required, p47^{phox}, p67^{phox} and the small GTP-binding protein Rac2. In the resting cell, these proteins are present in the cytosol and upon cellular activation associate with flavocytochrome b_{558} and convert it into a functional state in a process we have recently shown to be catalytic [4,5]. Genetic defects in any one of the p47^{phox}, p67^{phox}, p22^{phox} or gp91^{phox} proteins that result in the loss of their function (and failure of NADPH oxidase to generate O_2^-) cause a severe susceptibility to microbial infections, a syndrome known as chronic granulomatous disease (CGD) [6]. Recently, a description of a genetic defect in Rac2 resulting in neutrophil dysfunction and susceptibility to infection has been reported [7]. A further protein, p40^{phox}, has been implicated in oxidase activity, but to date its role has remained obscure. p40^{phox} was described independently by the groups of Segal and Someya, who identified and cloned a protein that was found to exist in a cytosolic complex with p67^{phox} and p47^{phox} [8,9]. These workers showed that p40^{phox} had significant sequence homology to p47^{phox} and p67^{phox} and that its expression was reduced markedly in the cytosols of CGD patients with p67^{phox} deficiency. Subsequent work showed that p40^{phox} becomes phosphorylated during neutrophil activation and translocates to the plasma membrane in a similar manner to p47^{phox} [10–13]. These findings have prompted extensive investigations into the molecular interactions of p47^{phox}, p67^{phox} and Rac with p40^{phox} [14–20]. p40^{phox} associates with p47^{phox} and p67^{phox} and is depleted in neutrophils of p67^{phox}-deficient CGD patients [8,21]. Studies of cytoskeletal associations of *phox* proteins indicate that p40^{phox} and p67^{phox} associate with the cytoskeletal fraction of resting cells, whereas p47^{phox} only becomes associated with the cytoskeleton during activation. p40^{phox} can dissociate from p67^{phox} however, possibly associating with p47^{phox}, both in whole cells and the cell-free system [11,22]. Despite these intensive studies into the role of p40^{phox}, little direct evidence for a function has been found for this protein to date, and it is not required for NADPH oxidase activity in the classical cell-free system for oxidase activation. Experiments overexpressing p40^{phox} in myeloid cell lines by Leto and co-workers [23] led them to conclude that p40^{phox} functions to down-regulate NADPH oxidase via interactions of SH3 domains within the *phox* proteins, a finding that was supported by inhibition of cell-free NADPH oxidase activity at high (greater-than-micromolar) p40^{phox} concentrations [23]. In contrast, Tsunawaki et al. [16] found that an antibody to the C-terminus of p40^{phox} dissociated p40^{phox} from the cytosolic *phox* complex and was inhibitory in the cell-free system, suggesting a positive role for p40^{phox} in oxidase activation. Here, for the first time, we show that under appropriate conditions p40^{phox} has a significant effect in enhancing the activation of NADPH oxidase in the cell-free system, probably by increasing the affinity of p47^{phox} for the enzyme.

EXPERIMENTAL

Reagents

All chemicals and reagents were of reagent grade and were from Sigma unless noted otherwise. Human neutrophils and their subcellular fractions were prepared and stored as described in [24]. Recombinant *phox* proteins were generated in Sf9 or Hi5

Abbreviation used: CGD, chronic granulomatous disease; O_2^-, superoxide.

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cells and purified to homogeneity as described previously [4]. Flavocytochrome b_{558} was purified from unstimulated neutrophil membranes, and relipidated and reflavinated as described earlier [25]. Flavocytochrome b_{558} concentrations were estimated from reduced-minus-oxidized difference spectra using an absorption coefficient of 21.6 $mM^{-1} \cdot cm^{-1}$ per mol of haem (10.8 $mM^{-1} \cdot cm^{-1}$ per mol of protein) [26].

Cell-free O_2^- assay

The rate of O_2^- production was measured using the superoxide dismutase-inhibitable rate of cytochrome *c* reduction in 96-well plates using a SpectraMax kinetic microtitre plate reader (Molecular Devices, Menlo Park, CA, U.S.A.) as described previously [5]. In brief, reaction mixtures contained 0.3 pmol of purified flavocytochrome b_{558} or 2×10^5 cell equivalents of neutrophil membranes (0.2–0.3 pmol of flavocytochrome b_{558}), 15 pmol of $p40^{phox}$, 15 pmol of $p47^{phox}$, 10 pmol of $p67^{phox}$ and 4 pmol of Rac2 in a final volume of 150 μl of Mg^{2+}-free relaxation buffer containing 100 μM cytochrome *c* [25]. Where stated, 2.5×10^6 cell equivalents of neutrophil cytosol were used in place of the recombinant cytosolic *phox* proteins. This amount of cytosol contains approx. 2.5 pmol of $p40^{phox}$, 15 pmol of $p47^{phox}$, 2.5 pmol of $p67^{phox}$ and 6.5 pmol of Rac2 (A. R. Cross and P. G. Heyworth, unpublished work). Oxidase activation was initiated by the addition of 100 μM SDS, and after 5 min of incubation the reaction was started by the addition of 160 μM NADPH. Each well was paired with an otherwise identical well supplemented with 300 units of superoxide dismutase. Assays were performed in duplicate or triplicate. Titration curves were fitted to a one-site binding equation (hyperbola) using GraphPad Prism software (GraphPad Prism, San Diego, CA, U.S.A.).

RESULTS AND DISCUSSION

$p40^{phox}$ increases the activity of the cell-free system using recombinant $p47^{phox}$, $p67^{phox}$ and Rac2 when employing neutrophil membranes, but not purified material, as a source of flavocytochrome b_{558}

During routine cell-free NADPH oxidase assays using recombinant cytosolic proteins ($p47^{phox}$, $p67^{phox}$ and Rac2), we have noted that the specific activity (turnover number) of neutrophil-membrane preparations is less than that expected when compared with highly purified flavocytochrome b_{558} preparations based on their respective flavocytochrome b_{558} contents (A. R. Cross and R. W. Erickson, unpublished work). In contrast, high specific activities were obtained using neutrophil cytosol in place of recombinant $p47^{phox}$, $p67^{phox}$ and Rac2, regardless of whether the source of flavocytochrome b_{558} was crude membranes or highly purified material. As part of our studies to identify the cause of this phenomenon, the effect of $p40^{phox}$ in the two systems was examined. Addition of $p40^{phox}$ to the system consisting of recombinant *phox* proteins and neutrophil membranes consistently gave a small (10–25 %) increase in activity (Table 1). In contrast, addition of $p40^{phox}$ had little effect on the recombinant system using purified flavocytochrome b_{558}, except at high concentrations where it appeared to have a mild inhibitory effect (results not shown). Leto and co-workers have reported similar inhibitory effects at high (greater-than-micromolar) concentrations of $p40^{phox}$ [23]. We hypothesize that this inhibitory effect may be due to sequesteration of one or more of the other *phox* components in a form where they cannot interact with their partner(s) in a normal fashion. $p40^{phox}$ was not capable of supporting significant amounts of O_2^- generation in the absence of $p47^{phox}$ (see below).

Table 1 $p40^{phox}$ increases the activity of NADPH in the cell-free system composed of neutrophil membranes and recombinant cytosolic proteins, but not in the system composed of neutrophil membranes and neutrophil cytosol

NADPH oxidase activity was measured in the cell-free system as described in the Experimental section. Where indicated, reaction mixtures contained 2×10^5 cell equivalents of neutrophil plasma membranes (Membranes), 2.5×10^6 cell equivalents of neutrophil cytosol (Cytosol), 15 pmol of $p40^{phox}$ and/or 15 pmol of $p47^{phox}$, 10 pmol of $p67^{phox}$ and 4 pmol of Rac2 (together referred to as Recombinant cytosol). The data represent one of three experiments performed in duplicate. Values are means ± S.E.M.

Reaction mixture	$p40^{phox}$	Specific activity (nmol of O_2^-/min per pmol of flavocytochrome b_{558})
Membranes	−	0.02 ± 0.03
Cytosol	−	0.001 ± 0.001
Recombinant cytosol	−	0.001 ± 0.0005
Membranes	+	0.02 ± 0.01
Membranes + cytosol	−	7.77 ± 0.125
Membranes + cytosol	+	7.84 ± 0.175
Membranes + recombinant cytosol	−	3.91 ± 0.024
Membranes + recombinant cytosol	+	5.30 ± 0.163

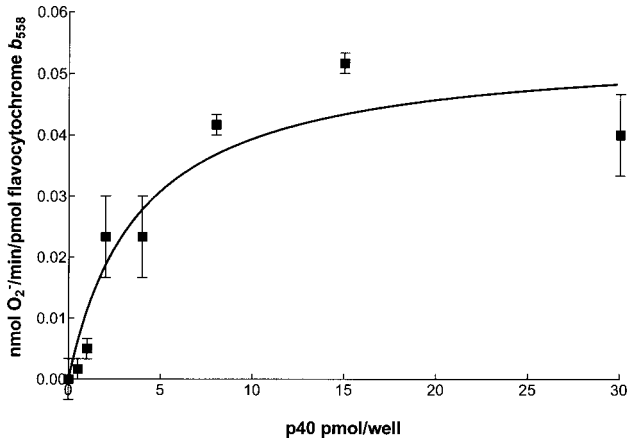


Figure 1 $p40^{phox}$ can only weakly activate NADPH oxidase in the absence of $p47^{phox}$

NADPH oxidase activity was measured in the cell-free system as described in the Experimental section. The reaction mixture contained 0.3 pmol of flavocytochrome b_{558}, 10 pmol of $p67^{phox}$ and 4 pmol of Rac2 and the indicated amount of $p40^{phox}$. The curve is the best fit to a one-site binding (hyperbola) equation calculated using GraphPad Prism. The data are representative of one of three experiments performed in duplicate. The error bars represent S.E.M.

$p40^{phox}$ cannot substitute for the other cytosolic *phox* components $p67^{phox}$ and Rac2

$p40^{phox}$ was incapable of supporting any O_2^--generating activity in the cell-free system using purified flavocytochrome b_{558} in the absence of either $p67^{phox}$ or Rac2 (results not shown), consistent with our previous findings [5,25]. In the absence of $p47^{phox}$, $p40^{phox}$ was capable of supporting an extremely low rate of O_2^- production, i.e. it could not effectively substitute for $p47^{phox}$ (Figure 1). Half-maximal effects were seen at 3.8 pmol of $p40^{phox}$/well (25 nM). It is suggested that this extremely small effect may be due to slightly increasing the affinity of $p67^{phox}$ and/or Rac2 for flavocytochrome b_{558} and mimicking the $p47^{phox}$-independent activity seen normally only at extremely high $p67^{phox}$

Table 2 p47^{phox} is the principal limiting component in the cell-free system composed of neutrophil membranes and 'recombinant cytosol'

NADPH oxidase activity was measured in the cell-free system as described in the Experimental section. Each reaction mixture contained 2×10^5 cell equivalents of neutrophil plasma membranes. Where indicated, the membranes were supplemented with 4×10^5 cell equivalents of neutrophil cytosol ('Spike'); 15 pmol of p47^{phox}, 10 pmol of p67^{phox} and 4 pmol of Rac2 (Recombinant cytosol); 15 pmol of p47^{phox}; 10 pmol of p67^{phox}; 4 pmol of Rac2; or 15 pmol of p40^{phox}. The data are representative of one of three experiments performed in duplicate. Values are means ± S.E.M.

Neutrophil membranes plus	Specific activity (nmol of O_2^-/min per pmol of flavocytochrome b_{558})
Cytosol	7.77 ± 0.125
'Spike'	0.865 ± 0.076
Recombinant cytosol	4.01 ± 0.024
'Spike' + recombinant cytosol	8.03 ± 0.024
'Spike' + p47^{phox}	7.4 ± 0.045
'Spike' + p67^{phox}	3.38 ± 0.044
'Spike' + Rac2	0.827 ± 0.055
'Spike' + p40^{phox}	1.91 ± 0.036

and Rac2 concentrations [4,27,28]. A maximum of 0.054 nmol of O_2^-/min per pmol of flavocytochrome b_{558} (compared with 15 nmol of O_2^-/min per pmol of flavocytochrome b_{558} or 0.4 %) was observed, with the maximum effect seen at 15 pmol/well (100 nM).

p47^{phox} is the principal limiting component in the cell-free assay for oxidase activation when neutrophil membranes are used in place of purified flavocytochrome b_{558}

The difference in responses to the recombinant cytosolic factors between neutrophil membranes and purified flavocytochrome b_{558} prompted the performance of experiments testing the effects of adding the recombinant proteins individually to neutrophil membranes that had been supplemented with a small, sub-optimal amount of neutrophil cytosol that alone was only capable of eliciting a low rate of O_2^- production. As can be seen in Table 2, the greatest amount of activity was recovered by the addition of p47^{phox} (15 pmol), suggesting that p47^{phox} is the principal limiting component in the system when neutrophil membrane, rather than purified material, is used as a source of flavocytochrome b_{558}. Some activity was also recovered by the addition of p67^{phox} (10 pmol), suggesting that it is partially limiting, whereas no further activity was obtained by the addition of Rac2 (4 pmol), suggesting that it is not limiting under these conditions. Significantly, some activity was also restored by the addition of p40^{phox} (15 pmol), and it is suggested that this is due to a co-operative interaction between p40^{phox} and p47^{phox}, described below, that increases the effective concentration of p47^{phox} present in the 'spike' of cytosol.

p40^{phox} functions by increasing the affinity of p47^{phox} for flavocytochrome b_{558}

The experiments described above suggested that the limiting cytosolic factor in the recombinant system employing neutrophil membranes was p47^{phox}. Since p40^{phox} augmented activity in this system, we hypothesized that p40^{phox} could promote the activity of p47^{phox} in some manner. Therefore, a series of experiments was designed to determine if p40^{phox} could increase the effectiveness of p47^{phox} in a cell-free system employing recombinant proteins and purified flavocytochrome b_{558}, under conditions where the limiting component was p47^{phox}. Decreasing the amount of p47^{phox} in the assay from 15 pmol (100 nM final concentration) to 1.5 pmol (10 nM) reduced the amount of O_2^- activity by about 75 %. Addition of 15 pmol of p40^{phox} (100 nM) restored the activity to approx. 75 % of that obtained with the near-saturating concentrations of p47^{phox} (Table 3). Increasing the p40^{phox} concentration 10-fold (Table 3) or 100-fold (results not shown) did not recover further activity. p40^{phox} did not exhibit a co-operative effect in similar experiments where p67^{phox} or Rac2 were the limiting components (results not shown).

Table 3 p40^{phox} augments the activity of p47^{phox} in the cell-free system of NADPH oxidase activation under conditions where p47^{phox} is limiting

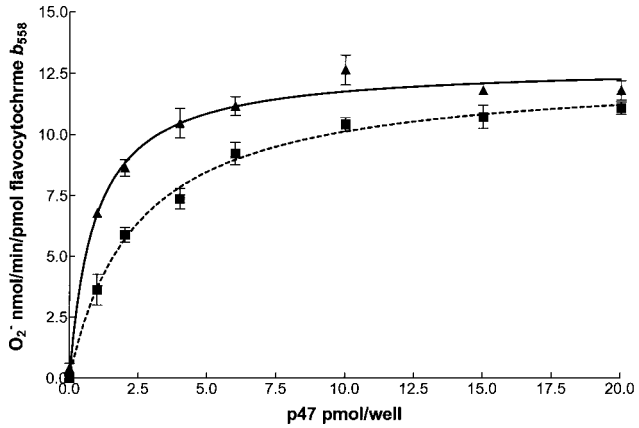
NADPH oxidase activity was measured in the cell-free system as described in the Experimental section. The reaction mixture contained 0.3 pmol of flavocytochrome b_{558}, 10 pmol of p67^{phox} and 4 pmol of Rac2, and the indicated concentrations of p40^{phox} and p47^{phox}.

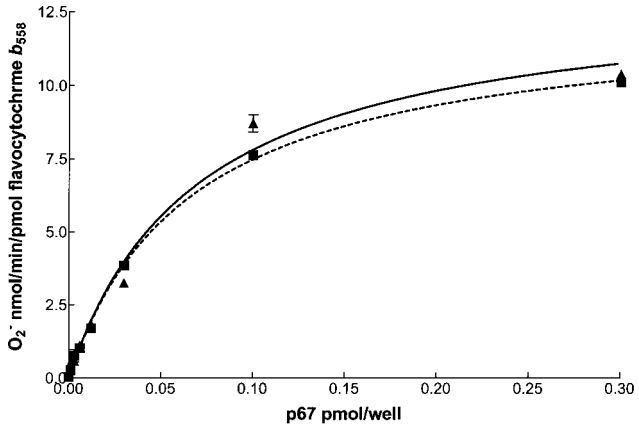
Flavocytochrome b_{558} + p67^{phox} + Rac2 plus	Specific activity (nmol of O_2^-/min per pmol of flavocytochrome b_{558})
100 nM p47^{phox}	12.17 ± 0.4
10 nM p47^{phox}	3.64 ± 0.41
10 nM p47^{phox} + 10 nM p40^{phox}	8.8 ± 0.43
10 nM p47^{phox} + 100 nM p40^{phox}	8.27 ± 0.37

In order to examine this co-operative effect in more detail, the concentration dependence of NADPH oxidase activity on p47^{phox} in the absence or presence of 100 nM p40^{phox} was measured (Figure 2, top panel). In the absence of p40^{phox}, half-maximal activity was observed at 2.4 ± 0.25 pmol/well (16 ± 1.7 nM final concentration), in the range that we have reported previously [5]. In the presence of p40^{phox} however, p47^{phox} was effective at significantly lower concentrations [half-maximal activity 0.9 ± 0.12 pmol/well (5.98 ± 0.78 nM final)]. The calculated V_{max} was similar in both cases, 12.55 and 12.81 nmol of O_2^-/min per pmol of flavocytochrome b_{558} in the absence and presence of p40^{phox} respectively, demonstrating that p40^{phox} increased the affinity of p47^{phox} in the activation process, but did not increase the overall activity. Similar experiments determining the concentration dependence of NADPH oxidase activity on p67^{phox} (Figure 2, middle panel) or Rac2 (Figure 2, lower panel) in the presence or absence of p40^{phox} did not show such co-operative effects, indicating that the effect was specific for p47^{phox}.

Concentration dependence of the co-operative effect of p40^{phox} on p47^{phox}

In order to estimate the concentration dependence of p40^{phox} in promoting the efficiency of p47^{phox} in activating NADPH oxidase, p40^{phox} was titrated into the cell-free system containing a sub-optimal amount of p47^{phox} as described above. The co-operative effect reached a plateau at relatively low concentrations of p40^{phox} (Figure 3), with a half-maximal response at 0.23 ± 0.05 pmol of p40^{phox}/well (1.5 ± 0.33 nM), increasing the activity from 3 nmol of O_2^-/min per pmol of flavocytochrome b_{558} to 6.56 nmol of O_2^-/min per pmol of flavocytochrome b_{558}. This concentration of p40^{phox} was much less than that present in neutrophil cytosol (460 nM; A. R. Cross and P. G. Heyworth, unpublished work). It is concluded that the function of p40^{phox} is, at least in part, to promote activation of NADPH oxidase by





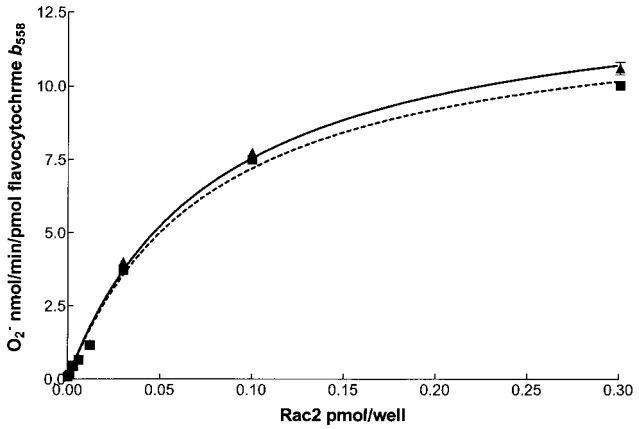


Figure 2 Concentration-dependence of NADPH oxidase activity on p47^{phox}, p67^{phox} and Rac2 in the absence and presence of p40^{phox}

NADPH oxidase activity was measured in the cell-free system as described in the Experimental section. The complete reaction mixture contained 0.3 pmol of flavocytochrome b_{558}, and 15 pmol of p47^{phox}, 10 pmol of p67^{phox} and 4 pmol of Rac2 with (▲) or without (■) 15 pmol of p40^{phox}. (Top panel) Increasing concentrations of p47^{phox} were added to the complete reaction mixture lacking p47^{phox}. (Middle panel) Increasing concentrations of p67^{phox} were added to the complete reaction mixture lacking p67^{phox}. (Bottom panel) Increasing concentrations of Rac2 were added to the complete reaction mixture lacking Rac2. The curves are the best fits to a one-site binding (hyperbola) equation calculated using GraphPad Prism. Each data set is representative of one of two or three experiments performed in duplicate. The error bars represent S.E.M.

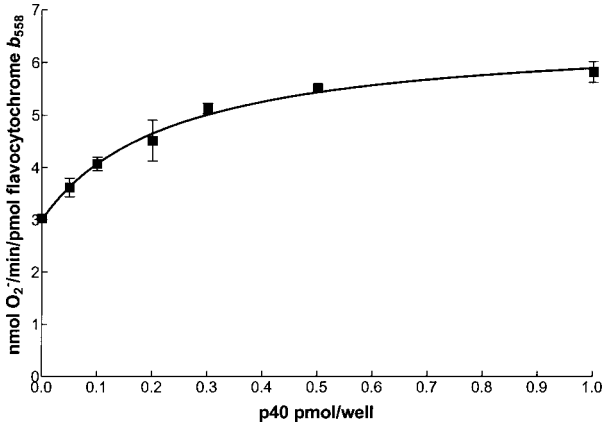


Figure 3 Concentration dependence of the co-operative effect of p40^{phox} on the ability of p47^{phox} to activate NADPH oxidase in the cell-free system

NADPH oxidase activity was measured in the cell-free system as described in the Experimental section. The reaction mixture contained 0.3 pmol of flavocytochrome b_{558}, 1.5 pmol of p47^{phox}, 10 pmol of p67^{phox}, 4 pmol of Rac2 and increasing concentrations of p40^{phox}. The curve is the best fit to a one-site binding (hyperbola) equation calculated using GraphPad Prism. The data represent one of two experiments performed in duplicate. The error bars represent S.E.M.

increasing the affinity of p47^{phox} for other oxidase components. In principle, this could be achieved in three ways. First, p40^{phox} could promote the conformational change in p47^{phox} that is believed for it to be necessary to bind to flavocytochrome b_{558} [29–31]. Secondly, p40^{phox} could bind to both flavocytochrome b_{558} and p47^{phox}, thus co-operatively increasing the binding affinity of the latter to the p40^{phox}–flavocytochrome b_{558} complex. Thirdly, p40^{phox} could bind to p67^{phox} and/or Rac2 in such a way as to increase the affinity of these components for p47^{phox}, thereby decreasing the concentration of p47^{phox} required to recruit them to flavocytochrome b_{558}. Distinguishing between these possibilities will be the focus of future work.

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