The neutrophil superoxide generating NADPH oxidase is activated by the assembly of cytosolic protein components with a membrane-associated flavocytochrome. The activity can be reconstituted in vitro using purified cytosolic factors p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac plus the phospholipid-reconstituted flavocytochrome b\textsubscript{558}. Here, we demonstrate that activity is reconstituted in the absence of p47\textsuperscript{phox} when high concentrations of p67\textsuperscript{phox} and Rac are used. V\textsubscript{max} values were the same in the presence or absence of p47\textsuperscript{phox}, yet p47\textsuperscript{phox} increases the affinity of both p67\textsuperscript{phox} and Rac for the oxidase complex by nearly 2 orders of magnitude. p67\textsuperscript{phox}(1-246), a truncated form of the protein which eliminates SH3 domains involved in binding to p47\textsuperscript{phox}, also supports superoxide generation, both in the presence and absence of p47\textsuperscript{phox}, providing further evidence for p47\textsuperscript{phox} independent activity. In the absence of p47\textsuperscript{phox}, p67\textsuperscript{phox}(1-246) binds to the NADPH oxidase complex 3-fold more tightly than does native p67\textsuperscript{phox} indicating that the C terminus contains a region which masks binding to the oxidase complex. Results indicate that p47\textsuperscript{phox} does not play a direct role in regulating electron transfer. Rather, its function is to serve as an adaptor protein to enhance the assembly of the other cytosolic components with the flavocytochrome and possibly to unmask a binding region in the N terminus of p67\textsuperscript{phox} by binding to its C-terminal domains. p67\textsuperscript{phox} and/or Rac play a more direct role in regulating electron transfer.

During the respiratory burst, neutrophils and other phagocytic cells produce superoxide and other reduced oxygen species that participate in microbial killing (1). The neutrophil superoxide generating NADPH oxidase catalyzes the enzymatic reduction of oxygen to produce superoxide. Upon activation, cytosolic components p47\textsuperscript{phox},\textsuperscript{1} p67\textsuperscript{phox} (2), and the small GTPase Rac translocate (3, 4) to membrane where they bind directly or indirectly in a multicomponent complex with flavocytochrome b\textsubscript{558}. The flavocytochrome b\textsubscript{558} contains all the prosthetic groups necessary for activity, FAD, two hemes, a consensus sequence for pyridine nucleotide binding (5, 6), but catalyzes the reduction of oxygen to superoxide only when assembled with the cytosolic components. Individuals with genetic deficiencies or mutations in p47\textsuperscript{phox}, p67\textsuperscript{phox} or one of the subunits of flavocytochrome b\textsubscript{558} (gp91\textsuperscript{phox} and p22\textsuperscript{phox}) exhibit chronic granulomatous disease, a condition marked by frequent infections as a result of the inability of neutrophils to generate superoxide and kill bacteria in vitro.

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\textsuperscript{1} The abbreviations used are: phox, phagocytic oxidase; GTP\textsuperscript{S}, guanosine 5’-O-(3-thiophosphate); PI, L-α-phosphatidylinositol; SM, sphingomyelin.

NADPH Oxidase Activity Is Independent of p47\textsuperscript{phox} in Vitro\*  

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RESULTS AND DISCUSSION

Previous reports have indicated that p47phox is necessary for NADPH oxidase activity in vivo (33–35) as well as in vitro (11, 12). As shown in the left half of Fig. 1 (filled bars), when relatively low concentrations of cytosolic proteins are used (i.e. submicromolar concentrations similar to those used in previous studies), p47phox, p67phox, and Rac all are needed to obtain a high rate of superoxide generation. These experiments used concentrations similar to those used in earlier studies and confirm published results. In contrast, when high concentrations of p67phox (6 μM) and Rac (2 μM) were used, a high rate of superoxide generation was seen even in the absence of p47phox (Fig. 1, open bars). p67phox and Rac are required, however. Even in the presence of very high concentrations of the other two components, little or no activity was seen when either of these components was omitted. Activity was specific for these NADPH oxidase components even at high concentrations. RhoA (2 μM), another member of the Rho family of small GTPases which is 59% identical to Rac, failed to replace Rac1 in this system. It was also possible that at high concentrations, p67phox was substituting for p47phox. Although these two proteins show rather low sequence identity overall (18%), they both contain two SH3 domains. It was therefore possible that the SH3 domains of p67phox at high concentrations were functioning in place of those of one of the cytosolic factors. Like p47phox and p67phox, Grb2 contains two SH3 domains. High concentrations of Grb2 (10 μM) failed to substitute for either p67phox or p47phox when normal concentrations of the remaining cytosolic factors were used (data not shown). p67phox-(1–246), a truncated version of p67phox lacking both SH3 domains, was also active at high concentrations in the NADPH oxidase assay system in the absence of p47phox (see below), indicating that the SH3 domains of p67phox were not substituting for those of p47phox.

To determine the extent to which the affinity for p67phox is influenced by p47phox, the concentration dependence of p67phox was determined in the presence and absence of p47phox (Fig. 2). The EC50 for p67phox was decreased 66-fold by p47phox (Table I). These results are consistent with our earlier report in which the EC50 value for p67phox varied inversely with p47phox concentration (21), except that the earlier study failed to achieve sufficiently high concentrations of p67phox to observe activity that was independent of p47phox. Despite the large change in p67phox EC50 values, the Vmax values were the same within experimental error (Table I). The effect of p47phox on the EC50 for p67phox is in agreement with previous studies indicating a role for p47phox in localizing p67phox to the NADPH oxidase complex (18). However, the generation of superoxide in the absence of p47phox clearly demonstrates that p67phox has one or more additional binding sites for the oxidase complex which is (are) independent of p47phox.

Further evidence for p47phox independent assembly of p67phox to the NADPH oxidase complex is seen in Fig. 3. p67phox-(1–246), which lacks both SH3 domains, was used in place of full-length p67phox. One or both of the SH3 domains of p67phox are known to mediate binding to a proline-rich region of p47phox (18–20). As shown, p67phox-(1–246) partially substituted for the full-length p67phox in supporting superoxide generation. Interestingly, in the absence of p47phox, truncated p67phox had a 3- to 4-fold lower EC50 than did full-length p67phox (see Table I). This may indicate that part of the function of the C-terminal half of the molecule is to mask or inhibit a binding domain in the N-terminal half. When this region is removed, the affinity of truncated p67phox for the NADPH oxidase is increased. Thus, in addition to acting directly to local-
NADPH Oxidase Activity Independent of p47phox

Superoxide generation was measured as described under "Experimental Procedures." The concentration dependence for Rac was determined in the presence and absence of p47phox. Although both truncated and full-length p67phox were added along with the indicated concentration of truncated p67phox or Rac, the rate of superoxide generation was measured. Kinetic parameters determined by Enzfitter are representative of three to four independent experiments. The experiments shown are representative of 3.

**TABLE I**

Effect of p47phox on kinetic parameters of Rac and p67phox

|        | EC50 (nm) | Vmax (nmol/min/pmol cytochrome b556) |
|--------|-----------|--------------------------------------|
| p67phox| +p47phox  | 24 ± 8                               |
|        | −p47phox  | 1590 ± 95                            |
| Truncated p67phox| +p47phox | 100 ± 20                             |
|        | −p47phox  | 460 ± 90                             |
| Rac    | +p47phox  | 47 ± 11                              |
|        | −p47phox  | 1613 ± 760                           |

**Fig. 2.** Concentration dependence of p67phox in the presence and absence of p47phox. Superoxide generation was as described under "Experimental Procedures." 476 nM Rac and 213 nM p47phox (A) or 2 μM Rac with no p47phox (B) were used along with the indicated concentration of p67phox and 10 nm cytochrome b556. Doubling the concentration of Rac had no effect on the p67phox EC50 in B, indicating Rac is saturating. Kinetic parameters determined by Enzfitter are 113 ± 8 nmol/min/pmol of cytochrome b556 (A) and 122 ± 5 nmol/min/pmol of cytochrome b556 (B) for Vmax and 24 ± 8 nm (A) and 1682 ± 250 nm (B) for EC50. In both experiments, Rac concentrations were sufficient to achieve greater than 85% of the Vmax. This experiment is representative of 3.

**Fig. 3.** Concentration dependence of truncated p67phox in the presence and absence of p47phox. 10 nm cytochrome b556, 2 μM Rac, and either 213 nm p47phox (A) or no p47phox (B) were added along with the indicated concentration of truncated p67phox, and the rate of superoxide generation was measured. Kinetic parameters determined by Enzfitter are 55 ± 5 nmol/min/pmol of cytochrome b556 (A) and 27 ± 5 nmol/min/pmol of cytochrome b556 (B) for Vmax and 85 nm ± 27 nm (A) and 374 ± 197 nm (B) for EC50. The experiment shown is representative of 3.

It is also possible that p47phox might induce its effects indirectly by altering the conformation of cytochrome b556.

To investigate the influence of p47phox on Rac, the concentration dependence for Rac was determined in the presence and absence of p47phox. Results (Fig. 4) were similar to those seen with p67phox; p47phox caused a 35-fold decrease in the EC50 for Rac, but did not alter the Vmax significantly (Table I). This decrease in EC50 could be due to a direct interaction between p47phox and Rac, or it could be mediated indirectly through p47phox binding to and altering the Rac association with another component of the oxidase. No direct interaction between Rac and p47phox has been observed in vitro (20, 23), but this remains a theoretical possibility. Alternatively, p47phox binding to cytochrome b556 or p67phox could induce a conformational change in one of these components, creating a higher affinity binding site for Rac. There is no direct evidence for binding of...
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Rac to the flavocytochrome, although in vivo translocation data have been interpreted in terms of such a complex (4). p67phox interacts with both p47phox and Rac (20, 23, 24). Therefore, the binding of p47phox to p67phox could induce a conformational change in the Rac binding site on p67phox, enhancing the binding of Rac. Based on current data, this seems to be the most likely mechanism, but this remains to be proven.

Since we were able to activate superoxide generation independently of p47phox, we tested the hypothesis that p47phox is the target of activation by arachidonate, as has been proposed by us and others (14, 36). Fig. 5 demonstrates that when high levels of p67phox and Rac are used, arachidonate is necessary for activation regardless of whether p47phox is present. Therefore, p47phox is either not the target of arachidonate activation or there is an additional component also influenced by arachidonate. Since the optimal concentration of arachidonate for activation is the same in the presence and absence of p47phox (data not shown) we favor the latter model in which p47phox is not the target of arachidonate. A possible target is cytochrome b556 since evidence also indicates that the proton channel activity associated with this transmembrane protein is dependent upon arachidonate (37). p67phox might also be the target for arachidonate, consistent with a kinetic study using SDS, another anionic amphiphilic activator of the oxidase (38).

An earlier study (25) indicated a role for p47phox in facilitat-

\[ \text{Rate (nmol/min/pmol cytochrome b556)} \]

\[ \text{[Rac] \text{ } \mu \text{M}} \]

\[ +p47-phox \]

\[ -p47-phox \]

**FIG. 4.** Concentration dependence of Rac in the presence and absence of p47phox. 150 nM p67phox and 213 nM p47phox (A) or 1.5 \mu M p67phox and no p47phox (B) were incubated with the indicated concentration of Rac and 10 nM cytochrome b556, and superoxide generation was measured as described under “Experimental Procedures.” Kinetic parameters determined by Enzfitter are 123 ± 15 nmol/min/pmol of cytochrome b556 (A) and 126 ± 22 nmol/min/pmol of cytochrome b556 (B) for $V_{\text{max}}$ and 54 ± 27 nm (A) and 1067 ± 122 nm (B) for $EC_{50}$. The experiment shown is representative of 3.

**FIG. 5.** Dependence of NADPH oxidase upon arachidonate. Superoxide generation was measured as in Fig. 1. Closed bars indicate the presence of 213 nM p47phox and the open bars indicate the absence of p47phox. Arachidonate (40 \mu M) was added as indicated. The experiment shown is representative of 4.

Superoxide generation was measured as in Fig. 1. Closed bars indicate the presence of 213 nM p47phox and the open bars indicate the absence of p47phox. Arachidonate (40 \mu M) was added as indicated. The experiment shown is representative of 4.

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\[ ^{3} \text{M. Kreck, unpublished observations.} \]
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Additions and Corrections

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Rac “insert region” is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65.

Jennifer L. Freeman, Arie Abo, and J. David Lambeth

Page 19797, Fig. 5: The values reported for the rate of superoxide generation in the purified cell-free NADPH oxidase system are 10 times the true values. This error does not affect the conclusions of the paper.

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NADPH oxidase activity is independent of p47phox in vitro.

Jennifer L. Freeman and J. David Lambeth

The values reported for the rate of superoxide generation in the purified cell-free system (Figs. 1–5 and Table I) are 10 times the true values. This error does not affect the conclusions of the paper.

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Structure/function analysis of the amino-terminal region of the α1 and α2 subunits of Na,K-ATPase.

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The α1M32 deletion mutant of the rat α subunit used in this study is identical to that described previously in Daly et al. (Daly, S. E., Lane, L. K., and Blostein, R. (1994) J. Biol. Chem. 269, 23944–23948) and was constructed so that the amino-terminal residue is the same (glycine) as that of the wild-type rat α1. Therefore, the sequence in Fig. 1 should read H_{2}N-MGVS--COOH, rather than H_{2}N-MEVSM--COOH as is shown. The deleted α1 (H_{2}N-MEVSM--COOH) has been expressed in HeLa cells. Its kinetic properties are similar to those of H_{2}N-MGVSM--COOH and, like H_{2}N-MGVSM--COOH, resemble those of α2 and α2(1-32α1).

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