The Cytosolic Component p47phox Is Not a Sine Qua Non Participant in the Activation of NADPH Oxidase but Is Required for Optimal Superoxide Production*

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The superoxide (O2−)-generating NADPH oxidase of phagocytes is a multicomponent complex consisting of a membrane-associated flavocytochrome (cytochrome b559), bearing the NADPH binding site and two redox centers (FAD and heme) and three cytosolic activating components: p47phox, p67phox, and the small GTPase Rac (1 or 2). The canonical view is that the induction of O2− generation involves the stimulus-dependent assembly of all three cytosolic components with cytochrome b559, a process mimicked in vitro by a cell-free system activated by anionic amphiphiles. We studied the requirement for individual cytosolic components in the activation of NADPH oxidase in a cell-free system consisting of purified and relipidated cytochrome b559, recombinant p47phox, p67phox, and Rac1, and the amphiphile, lithium dodecyl sulfate. We found that pronounced activation of NADPH oxidase can be achieved by exposing cytochrome b559 to p67phox and Rac1, in the total absence of p47phox (turnover = 60 mol O2−/mol cytochrome b559). However, maximal activation (turnover = 153 mol O2−/mol cytochrome b559) could only be obtained in the presence of p47phox. O2− production, in the absence of p47phox, was dependent on: high molar ratios of p67phox and Rac1 to cytochrome b559, Rac1 being in the GTP-bound form, cytochrome b559 being saturated with FAD, and an optimal concentration of amphiphile. Single cytosolic components or combinations of two cytosolic components, other than p67phox and Rac1, were incapable of activation. We conclude that p67phox and Rac1 are the only cytosolic components directly involved in the induction of electron transport in cytochrome b559-p47phox appears to facilitate or stabilize the interaction of p67phox and, possibly, Rac1 with cytochrome b559 and is required for optimal generation of O2− under physiological conditions.

The production of oxygen radicals, in response to triggering of cell-surface receptors, is the paramount microbicidal mechanism of phagocytic cells. The primary reaction in this process is the generation of superoxide (O2−), derived by the one-electron reduction of molecular oxygen, catalyzed by a membrane-bound heterodimeric flavocytochrome (cytochrome b559), utilizing NADPH as the source of electrons (reviewed in Refs. 1–3). Electron flow from NADPH to oxygen is activated consequent to the interaction of cytochrome b559 with three cytosolic proteins, p47phox, p67phox, and the small GTPase Rac (1 or 2), leading to the formation of a multimolecular structure, known as the NADPH oxidase complex (reviewed in Ref. 4). The elicitation of an oxidative burst in intact phagocytes is accompanied by the translocation to the plasma membrane of about 10% of p47phox and p67phox (5). Translocation of p47phox to the membrane is linked to the phosphorylation of the component at multiple sites (6). Also, movement of p47phox and p67phox to the membrane requires the presence of cytochrome b559 (7). It is of interest that membrane localization of p67phox is dependent on p47phox (7), suggesting that at least one of the functions of p47phox is to escort p67phox to the membrane. Whether activation or an oxidative burst is associated with translocation of Rac to the membrane is still controversial (8, 9). A number of studies dealt with the interaction of cytosolic components with the cytoskeleton (10, 11). Of special relevance to the subject of the present communication is the finding that, in preparations obtained from both resting and stimulated leukocytes, p47phox is detected in the soluble fraction, whereas p67phox is associated with the cytoskeleton, which is also the site of O2− production (11).

The activation of NADPH oxidase can be mimicked in vitro by a cell-free system consisting, in its most basic form, of membranes and the cytosolic fraction of resting phagocytes exposed to a critical concentration of some anionic amphiphiles (12–16). Complete reconstitution of a functioning NADPH oxidase can be achieved in cell-free systems consisting exclusively of highly purified or recombinant components (17–19). The most commonly utilized version of this is the semi-recombinant system, consisting of purified relipidated and reflavinated cytochrome b559 combined with recombinant p47phox, p67phox, and Rac (18). It is commonly accepted that the cell-free system contains all the basic components necessary for NADPH-dependent O2− generation, but is lacking at least one cytosolic component, probably active in the intact cell (p40phox) (20), and all signal transduction and regulatory elements. Experimental evidence has, so far, supported the canonical view that NADPH oxidase activation in the cell-free system is an “all or none” phenomenon, in the sense that all three cytosolic components, in rigorous molar proportions, are required for the activation of electron flow across the redox centers of cytochrome b559. In spite of recent advances in our understanding of the nature of protein-protein interactions among components, leading to the assembly of the NADPH oxidase complex (reviewed in Ref. 4),

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the molecular basis of the ensuing activation of electron transport in cytochrome b_{559} remains obscure.

Three pieces of evidence suggest that not all cytosolic components are directly involved in the “activation” of cytochrome b_{559} or that individual components might have distinctive roles in this process. First, O_2^− production could be induced in solubilized membranes (21) or purified and repurified cytochrome b_{559} (22, 23) by phosphatidic acid, in the absence of cytosolic components. Second, the finding (11) that p47^{phox} was absent from the cytoskeletal fraction of activated neutrophils, which contained the bulk of O_2^− forming activity, was difficult to reconcile with the concept that p47^{phox} is more than a transient constituent of the NADPH oxidase complex. Finally, Cross et al. (24) demonstrated that the activated NADPH oxidase possessed a diaphorase activity, which was dependent on p67^{phox} but not on p47^{phox}. They went on to show that the two cytosolic components have unequal roles in the regulation of electron flow in cytochrome b_{559}, p67^{phox} stimulated the flow from NADPH to FAD, whereas p47^{phox} was required for electron transport from FAD to heme and oxygen (25).

The subject of this paper is the finding that purified repurified and relipidated cytochrome b_{559} generates high amounts of O_2^− when combined in vitro with p67^{phox}, Rac1, and an anionic amphiphile, in the total absence of p47^{phox}.

**EXPERIMENTAL PROCEDURES**

**Purification of Cytochrome b_{559}**—Cytochrome b_{559} was purified from guinea pig peritoneal macrophage membranes, solubilized by n-octyl-β-D-glucopyranoside (26), by a recent modification (27) of the original procedures (22, 28), and had a specific content of 4–6 nmol of heme/mg of protein. The material was concentrated by ultrafiltration to a concentration of 2–3 μM and relipidated with 200 μM l-α-phosphatidylcholine (type IV-S, Sigma) by dialysis against detergent-free buffer (27). The material was found to be free of possible contamination by cytosolic components by immunoblotting with polyclonal antibodies raised against purified recombinant p47^{phox} and p67^{phox} (29) (kind gifts of Dr. Thomas L. Leto) and with a polyclonal antibody to Rac1 (see 21, Santa Cruz Biotechnology).

**Preparation of Recombinant Cytosolic NADPH Oxidase Components—p47^{phox} and p67^{phox}** were prepared in Sf9 cells, infected with baculoviruses carrying cDNAs for human cytosolic components (kind gifts of Dr. Thomas L. Leto), by a modification of the procedure described by Leto et al. (29). The differences from the original procedure were: the Sf9 cells were grown as suspension cultures in serum-free medium (SF-900 II SFM, Life Technologies); the cells were disrupted by sonication; purification of p67^{phox} on Q-Sepharose was done at pH 7.5, and purification of p47^{phox} by cation exchange chromatography, was performed on SP-Sepharose (HighLoad 16/10, Pharmacia Biotech Inc.). The purified recombinant proteins were analyzed by SDS-polyacrylamide gel electrophoresis; p67^{phox} was 90% pure, whereas p47^{phox} was 99% pure. Both p67^{phox} and p47^{phox} were recognized, in an enzyme-linked immunosorbent assay (30), by antibodies to p67^{phox} and p47^{phox} (29), respectively, and no evidence was found, by this methodology, for cross-contamination between the two recombinant proteins. Recombinant Rac1 was isolated from Escherichia coli transformed with Rac1 cDNA subcloned into the bacterial expression vector pGEX2T (kind gifts of Dr. Thomas L. Leto), as described by Kwong et al. (31). The preparation was found to be 99% pure and free of contamination by p47^{phox} and p67^{phox}, as tested by enzyme-linked immunosorbent assay.

**Nucleotide exchange on Rac1** to guanosine 5′-(thio)triphosphate (GTP-S) was performed as described previously (32).

**Functional Assays**—For the measurement of amphiphile-activated O_2^− production, purified repurified cytochrome b_{559} was diluted to a final concentration of 0.05–0.08 μM in assay buffer consisting of 65 mM Na-K-phosphate, pH 7.0, 1 mM MgCl_2, 1 mM EGTA, and 2 mM NaN_3. To the buffer were added FAD, in a 3:1 molar ratio to cytochrome b_{559}, lithium dodecyl sulfate (LiDS), at a concentration of 100–180 μM, and various combinations of p47^{phox}, p67^{phox}, and Rac1-GTP-S in molar ratios to cytochrome b_{559} varying from 1:1 to 80:1. The activation mixture, with a total volume of 50 μl, was incubated for 3 min at room temperature, and O_2 production was assessed by transferring it to 0.95 ml of assay buffer, supplemented with 100 μM ferricytochrome c, 100 μM LiDS, and 200 μM NADPH, and measuring the kinetics of cytochrome c

![Fig. 1. O_2 production by a cell-free system in the absence of p47^{phox}.](image)

**RESULTS AND DISCUSSION**

We found that O_2^− was generated by a cell-free system consisting of purified and repurified cytochrome b_{559} (0.073 μM) and various combinations of recombinant cytosolic components, at the following concentrations: p67^{phox} (2.96 μM); Rac1-GTP-S (1 μM), and p47^{phox} (0.88 μM). The concentration of LiDS in the primary activation mixture was 112 μM. Tracings represent kinetics of cytochrome c reduction in the presence of the following combinations of cytosolic components: trace 1, p47^{phox} + p67^{phox} + Rac1-GTP-S; trace 2, p67^{phox} + Rac1-GTP-S; trace 3, p47^{phox} + p67^{phox}; trace 4, p47^{phox} + Rac1-GTP-S; trace 5, p67^{phox}. The arrow indicates the time of addition of the 50-μl reaction mixture to the 0.95-ml assay buffer, containing NADPH. The figure illustrates one out of four similar experiments.

**Fig. 1.** O_2 production by a cell-free system in the absence of p47^{phox}. O_2 production was elicited, as described under “Experimental Procedures,” in a cell-free system consisting of purified and repurified cytochrome b_{559} (0.073 μM) and various combinations of recombinant cytosolic components, at the following concentrations: p67^{phox} (2.96 μM); Rac1-GTP-S (1 μM), and p47^{phox} (0.88 μM). The concentration of LiDS in the primary activation mixture was 112 μM. Tracings represent kinetics of cytochrome c reduction in the presence of the following combinations of cytosolic components: trace 1, p47^{phox} + p67^{phox} + Rac1-GTP-S; trace 2, p67^{phox} + Rac1-GTP-S; trace 3, p47^{phox} + p67^{phox}; trace 4, p47^{phox} + Rac1-GTP-S; trace 5, p67^{phox}. The arrow indicates the time of addition of the 50-μl reaction mixture to the 0.95-ml assay buffer, containing NADPH. The figure illustrates one out of four similar experiments.
TABLE I

Composition of cell-free system

| Composition                  | Turnover
|------------------------------|----------
| Cytochrome b_{559} + p47_{phox} + p67_{phox} + Rac1-GTP·S$_{p}$ | 153.5 ± 18.0 (n = 4) |
| Cytochrome b_{559} + p67_{phox} + Rac1-GTP·S$_{p}$ | 60.3 ± 4.3 (n = 4) |
| Cytochrome b_{559} + p47_{phox} + p67_{phox} + Rac1-GTP·S$_{p}$ | 2.9 ± 0.6 (n = 3) |
| Cytochrome b_{559} + p47_{phox} + Rac1-GTP·S$_{p}$ | 2.0 ± 0.2 (n = 4) |
| Cytochrome b_{559} + p67_{phox} + Rac1-GTP·S$_{p}$ | 1.8 |
| Cytochrome b_{559} + Rac1-GTP·S$_{p}$ | 2.6 |

Note: Turnover rates are significant from those measured in the complete system (p < 0.005).

* Cytochrome b_{559}, 12:1, p67_{phox}, 41:1, and Rac1-GTP·S$_{p}$, 14:1.
* p67_{phox}, 12:1 and Rac1-GTP·S$_{p}$, 14:1. Turnover rates are significantly different from those measured in the complete system and in the system consisting of cytochrome b_{559} + p67_{phox} + Rac1-GTP·S$_{p}$ (p < 0.001, for both comparisons).
* p47_{phox}, 12:1, Rac1-GTP·S$_{p}$, 14:1. Turnover rates are significantly different from those measured in the complete system and in the system consisting of cytochrome b_{559} + p67_{phox} + Rac1-GTP·S$_{p}$ (p < 0.001, for both comparisons).

Fig. 2. Dose-response curves of p67_{phox} and Rac1-GTP·S$_{p}$ in the cell-free O$_{2}$-generating system, lacking p47_{phox}. O$_{2}$ production was elicited as described under "Experimental Procedures." The concentration of LiDS in the primary activation mixture was 112 μM. A, the cell-free system consisted of purified and relipidated cytochrome b_{559} (0.073 μM), 1 μM Rac1-GTP·S$_{p}$, and varying concentrations of p67_{phox} (0.96–6.037 μM). B, the cell-free system consisted of purified and relipidated cytochrome b_{559} (0.073 μM), 3.02 μM p67_{phox}, and varying concentrations of Rac1-GTP·S$_{p}$ (0.167–2.01 μM). The data are derived from one experiment.

**requirement for activation; none of the two components was active by itself and exposure of cytochrome b_{559} to only one component during the 3-min incubation in 50 μl followed by the dialysis of the second component together with the 0.95 ml of assay buffer, did not lead to O$_{2}$ generation. Neither of the three cytosolic components added alone nor combinations of two cytosolic components, other than p67_{phox} and Rac1, at molar ratios in relation to cytochrome b_{559} of up to 40:1, were capable of NADPH oxidase activation.

As seen in Table I, turnover rates reaching 40% of those obtained in the complete cell-free system, were achieved by activation of cytochrome b_{559} with the combination of p67_{phox} and Rac1, whereas single cytosolic components or all other combinations of two cytosolic components elicited turnover rates of less than 1.9% of those measured in the complete system. Fig. 2 illustrates the dose-response dependence of O$_{2}$ production, in the p47_{phox}-deficient cell-free system, on the concentration of p67_{phox} (A) and Rac1 (B) in the presence of a constant amount of cytochrome b_{559} and a saturating amount of the second cytosolic component. It is apparent that maximal O$_{2}$ production is obtained at molar excesses over cytochrome b_{559} of 40:1, for p67_{phox} and 15:1 for Rac1.

The lipid composition of the cytochrome b_{559} liposomes utilized in the present experiments was identical to that described in our original reports on the methodology of cytochrome b_{559} relipidation (26, 28) and to that of liposomes described in other reports on the semirecombinant cell-free system, in which an absolute requirement for three cytosolic components was demonstrated (17, 19).

These results are best explained by a novel interpretation of the mechanism of NADPH oxidase assembly. We propose that the induction of electron flow in cytochrome b_{559} is mediated by the cooperative action of p67_{phox} and Rac1, in the GTP-bound state, and that p47_{phox} is not directly involved in this process. This proposal is compatible with a number of earlier findings. These are: (a) p67_{phox} is found in the cytosol of phagocytes as a complex with p47_{phox} (33, 34), and it was suggested that they may translocate en bloc to the plasma membrane (34); (b) translocation of p67_{phox} to the cell membrane is absolutely dependent on the presence of p47_{phox} (7); (c) protein-protein interactions between p67_{phox} and p47_{phox} have been shown to be mediated by bidirectional affinities of SH3 domains on one component for proline-rich domains on the other component (35–40), and, recently, also by what appears to be a charge-related mechanism (41). Whereas some of these interactions are constitutive, others are enhanced by stimuli eliciting O$_{2}$ generation (40); (d) stimulus-dependent translocation of p67_{phox} to the membrane in intact cells requires the interaction of its NH$_{2}$-terminal domain (residues 1–246) with the two SH3 domains of p47_{phox} (40). It is of interest that the same aminoterminal domain of p67_{phox}, which lacks both SH3 domains, is sufficient for cell-free NADPH oxidase activation (36); (e) whereas there is an abundance of data on the molecular basis of the interaction between p47_{phox} and cytochrome b_{559}, involving SH3 (36–38, 40) and other (42–45) domains, the possibility of an interaction between p67_{phox} and cytochrome b_{559} does not seem to have been investigated. The only exception is the
report by Uhlinger et al. (46), proposing, on the basis of kinetic analysis of NADPH oxidase activation in a semirecombinant cell-free system, that p67<sub>phox</sub> interacts directly with cytochrome b<sub>559</sub> (f) there is evidence (47, 48) for constitutive binding of Rac1 to the amino-terminal region of p67<sub>phox</sub>, a domain also involved in stimulus-dependent interaction with the SH3 domains of p47<sub>phox</sub>, and it was recently shown that p67<sub>phox</sub> is required for the stimulus-elicited translocation of Rac1 to the membrane (49); (g) finally, indirect evidence was presented for an interaction between Rac2 and cytochrome b<sub>559</sub> by the finding of reduced membrane localization of Rac2 in cells of chronic granulomatous disease (CGD) patients lacking cytochrome b<sub>559</sub> (50).

We conclude that, in a cell-free setting, a marked level of NADPH oxidase activation can be achieved in the absence of p47<sub>phox</sub> (and Rac1?) with cytochrome b<sub>559</sub> is either of low affinity or possesses a high dissociation rate. p47<sub>phox</sub>, by virtue of its ability to bind to both cytochrome b<sub>559</sub> and p67<sub>phox</sub>, by molecular affinities enhanced by

5 NADPH oxidase activation can be achieved in the absence of
559 and p67<sub>phox</sub> in vitro

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