Activation of the Leukocyte NADPH Oxidase by Protein Kinase C in a Partially Recombinant Cell-free System

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Lucia Rossetti Lopes‡§, Carolyn R. Hoyal‡§, Ulla G. Knaus‡, and Bernard M. Babior‡

From the ‡Department of Molecular and Experimental Medicine and the §Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

The NADPH oxidase is a membrane-associated enzyme that catalyzes the one electron reduction of oxygen to O$_2^-$ at the expense of NADPH. A correlation between the activation of the oxidase and the phosphorylation of p47$^{PHOX}$, a cytosolic oxidase component, is well recognized in whole cells, and direct evidence for a relationship between the phosphorylation of this oxidase component and the activation of the oxidase has been obtained in a number of cell-free systems containing neutrophil membrane and cytosol. Using superoxide dismutase-inhibitable cytochrome c reduction to quantify O$_2^-$ production, we now show that p47$^{PHOX}$ phosphorylated by protein kinase C activates the NADPH oxidase not only in a cell-free system containing neutrophil membrane and cytosol, but also in a system in which the cytosol is replaced by the recombinant proteins p67$^{PHOX}$, Rac2, and phosphorylated p47$^{PHOX}$, suggesting that neutrophil plasma membrane plus these three cytosolic proteins are both necessary and sufficient for oxidase activation. In both the cytosol-containing and recombinant cell-free systems, however, activation by SDS yielded greater rates of O$_2^-$ production than activation by protein kinase C-phosphorylated p47$^{PHOX}$, indicating that a system that employs protein kinase C-phosphorylated p47$^{PHOX}$ as the sole activating agent, although more physiological than the SDS-activated system, is nevertheless incomplete.

The NADPH oxidase is a membrane-associated enzyme that catalyzes the one electron reduction of oxygen to O$_2^-$ at the expense of NADPH (1). The oxidase comprises multiple protein components present in both the cytosol and the plasma membrane. The enzyme is dormant in resting cells but becomes activated when the cells are exposed to appropriate stimuli. Upon activation, a cytosolic complex consisting of the oxidase components p47$^{PHOX}$, p67$^{PHOX}$, and p40$^{PHOX}$ associates with the membrane-bound cytochrome b$_{558}$ to assemble the active oxidase (2–7).

The phosphorylation of p47$^{PHOX}$ is a well recognized concomitant of oxidase activation in whole cells, but the mechanism of activation of the oxidase is not fully understood (8–14). One key to understanding the activation of the oxidase emerged with the discovery of the cell-free activation system (15–17) in which it was shown that NADPH oxidase activity could be induced in a mixture of membrane and cytosol by the addition of amphiphiles like arachidonic acid (15, 17) and SDS (16, 18). Recently, increasing attention has been paid to cell-free systems in which the oxidase is activated without using anionic amphiphiles (19–22). Our studies showed that the oxidase can be activated by p47$^{PHOX}$ phosphorylated by protein kinase C in a cell-free system containing neutrophil membrane and cytosol (21). In addition, these studies also revealed that the phosphorylation of p47$^{PHOX}$ was not the only ATP-dependent step in the activation of the oxidase by protein kinase C. A preceding phosphorylation event occurs in the membranes rendering them capable of supporting oxidase activation. The target of this event has yet to be determined. Although these experiments showed a direct relationship between the phosphorylation of p47$^{PHOX}$ and the activation of the oxidase, the use of whole cytosol made it difficult to recognize whether cytosolic factors other than those necessary for activation by SDS are required for oxidase activation by a kinase. In this paper we report studies of a recombinant cell-free system containing only membrane and cytosolic oxidase components (p47$^{PHOX}$, p67$^{PHOX}$, and Rac2). Our findings suggest that the cytosolic components phosphorylated p47$^{PHOX}$, p67$^{PHOX}$, and Rac2 are sufficient for partial activation of the oxidase.

EXPERIMENTAL PROCEDURES

Materials—Chemicals and enzymes were obtained from the following sources: dextran and Ficoll-Hypaque from Amersham Pharmacia Biotech; phosphatidylycerine, diacetylglycerol, isopropyl-$b$-thiogalactopyranoside, NADPH, ATP, guanosine 5'-O-(3-thiotriphosphate) (GTP$^\gamma$S),$^1$ guanosine 5'-O-(2-thiodiphosphate) (GDP$^\delta$S), glutathione agarose and cytochrome c from Sigma; rat brain protein kinase C, calcinulin A, and GF-109203X from Calbiochem; and the Bradford protein assay reagent from Bio-Rad.

Preparation of Neutrophil Fractions—Neutrophil cytosol and membrane were prepared as described by Borregaard et al. (23). Neutrophils were prepared from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-anticoagulated blood. The neutrophils were suspended at 10$^6$ cells/ml in a modified relaxation buffer (0.1 M KCl, 3 mM NaCl, 3.5 mM MgCl$_2$, 10 mM PIPES buffer, pH 7.3). Plasma membrane and cytosol were prepared by nitrogen cavitation followed by centrifugation through a Percoll gradient. Both cytosol and membrane were divided into aliquots and stored at $-70^\circ$C until use.

Production and Purification of Recombinant p67$^{PHOX}$ from Baculovirus-infected S9 Cells—Purified recombinant p67$^{PHOX}$ was produced by means of the baculovirus system described by Leto et al. (1991), using a p67$^{PHOX}$-expressing recombinant virus generously provided by T. L. Leto. Large scale production of pure recombinant p67$^{PHOX}$ was achieved by infecting monolayer cultures of S9 cells in 150 cm$^2$ flasks at a density of 1–2 × 10$^6$ cells/ml (24). Cells were harvested 72 h postinfection, washed twice in phosphate-buffered saline by centrifugation at 400 × g for 10 min, and then resuspended to 5 × 10$^7$/ml in lysis

$^1$ The abbreviations used are: GTP$^\gamma$S, guanosine 5'-O-(3-thiotriphosphate); GDP$^\delta$S, guanosine 5'-O-(2-thiodiphosphate); PAGE, polyacrylamide gel electrophoresis.
buffer (50 mM KCl, 3 mM NaCl, 2 mM MgCl₂, 0.1 mM dithiorethiol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 5.4 mM PIPES, pH 7.5). All subsequent work was conducted at 4 °C. Cells were disrupted by sonication (4 x 10 s) and centrifugation at 400 g for 10 min. The supernatant fraction containing p67PHOX was brought to 45% saturation with solid ammonium sulfate. The resulting precipitate was isolated by centrifugation (12,000 g for 30 min), then dissolved in 10 ml of buffer A (20 mM Tris, pH 7.5, 0.1 mM dithiorethiol, 1 mM EDTA, 2 mM EGTA, 0.15 mM phenylmethylsulfonyl fluoride) and dialyzed overnight against the same buffer. The dialyzed solution was applied to a Mono Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) previously equilibrated with buffer A and washed with 5 volumes of the same buffer. Proteins were eluted from the column by fast protein liquid chromatography with a 0.1–0.3 M NaCl gradient in the same buffer at a flow rate of 0.8 ml/min. The fractions containing purified p67PHOX were pooled and stored at −70 °C.

Preparation of Recombinant GST-p47PHOX and Rac2 Fusion Proteins—Recombinant fusion proteins composed of glutathione S-transferase (GST) linked downstream to p47PHOX or Rac2 were isolated from *Escherichia coli* transformed with pGEX-1 plasmids containing cDNA inserts encoding the downstream proteins as described by Park et al. (3). The fusion proteins were purified by affinity chromatography on glutathione-agarose beads. Initially the culture was grown overnight at 37 °C to 100 ml of “Terrific Broth” containing 0.1% ampicillin, then diluted into 1 liter of fresh Terrific Broth/ampicillin. The diluted cultures were grown for an additional hour at 37 °C (for GST-p47PHOX expression) or an additional 2.5 h at 37 °C (for GST-Rac2 expression). Isopropyl-β-thiogalactopyranoside (0.1 mM) was then added, and the cultures were grown with vigorous agitation for an additional 3 h at 37 °C for GST-p47PHOX expression or 30°C for GST-Rac2 expression. At the conclusion of the incubations, the cells were recovered by centrifugation at 2000 g for 10 min at 4 °C. The GST-p47PHOX pellet was suspended in 10 ml of ice-cold phosphate-buffered saline containing a 1 x mixture of protease inhibitors (Roche Molecular Biochemicals), while the GST-Rac2 pellet was resuspended in a lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiorethiol, and 1 mM phenylmethylsulfonyl fluoride). The cells were then disrupted by sonication. The sonicates were clarified by centrifugation at 14,000 x g for 15 min at 4 °C. The fusion proteins were isolated from the supernatant by purification over glutathione-agarose as described by Smith and Johnson (25). Before use, excess glutathione was removed from the solution of purified recombinant protein by dialysis against relaxation buffer. The concentrations of all proteins (95–99% pure) were determined with a Bio-Rad assay kit using bovine serum albumin as a standard.

Phosphorylation of GST-p47PHOX—Phosphorylation of recombinant GST-p47PHOX was carried out using 200 μg of fusion protein in final volume of 200 μl. The reaction mixture contained 1 mM ATP, 10 mM magnesium acetate, 1.0 mM CaCl₂, 10 μg phosphatidylserine, 1 μg diacylglycerol, and 0.5 unit of protein kinase C (in 200 μl of relaxation buffer, 0.1 x KCl, 3 mM MgCl₂, 3.5 mM MgCl₂, 10 mM PIPES buffer, pH 7.3). The lipids were added as mixed liposomes prepared by dissolving 2.5 mg/ml phosphatidylserine and 1 mg/ml diacylglycerol in chloroform, removing the chloroform under a stream of nitrogen, and then sonating the dried lipids for 2 min on ice in 0.8 ml of 20 mM Tris buffer, pH 7.4. Incubations were carried out for 30 min at 37 °C. The phosphorylated protein, designated p47PHOX-P, 2 was separated from the reaction mixture as described elsewhere (21).

Cell-free Activation of the NADPH Oxidase with p47PHOX-P 2—Activation of the NADPH oxidase in the cell-free system was directly measured by following the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm in a dual beam recording spectrophotometer. The complete reaction mixture contained 5 x 10⁶ cell equivalents of membrane (12 ± 1.4 pmol of cytochrome b₅₅₉) incubated for 10 min at 30 °C with 50 μM GTPγS or GDPβS, 1 mM ATP, 250 mM cayucin A, and 105 pmol of GST-Rac2, 75 pmol of p67PHOX and 70 pmol of GST p47PHOX, phosphorylated or unphosphorylated, in a final volume of 200 μl. GST-Rac2 was reconstituted with 130 μM GTPγS or by preincubation of 105 pmol of the protein with 2.6 mM EDTA for 10 min at room temperature by the addition of 25 μM MgCl₂ (26). Reactions were started by adding the detection mixture (0.1 mM cytochrome c and 0.16 mM NADPH, final concentrations). Reduction was followed in a Uvikon 941 dual beam recording spectrophotometer (Kontron Instru-

**FIG. 1. Recombinant proteins used in these experiments.** SDS-PAGE of the purified proteins was performed as described under “Experimental Procedures,” using a 10% running gel. Coomassie Blue staining was used to show GST-p47PHOX (lane 1), p67PHOX (lane 2), and GST-Rac2 (lane 2).

**Protein kinase C-phosphorylated P47**

Protein kinase C-phosphorylated P47PHOX is designated p47PHOX-P, because it contains 6 mol of phosphate/mol of p47PHOX.
which we supplemented the cell-free system with p47PHOX (added as the GST fusion protein), we found that the enzyme could be activated without detergent, provided the p47PHOX was first phosphorylated by protein kinase C (21). We believe that the activation of the cell-free oxidase by protein kinase C may represent a more physiological process than activation by anionic amphiphiles, because in intact cells, as in the kinase-activated cell-free system, oxidase activation is associated with the phosphorylation of p47PHOX.

It has been shown by others that in a cell-free system in which neutrophil cytosol has been replaced by the two recombinant cytosolic oxidase subunits p47PHOX and p67PHOX together with the small GTPase Rac2, O2 is produced upon the addition of SDS (30). In order to see if the same system could be activated by a kinase, we conducted experiments in which O2 production was measured in a recombinant system that contained phosphorylated p47PHOX (i.e. p47PHOX-P0) instead of the unphosphorylated protein. The results (Fig. 2) showed that O2 was produced in the complete system, but that the omission of all or any one of the three recombinant cytosolic proteins or the omission of membrane (not shown) essentially eliminated oxidase activity. The use in the recombinant system of Rac2 preloaded with GDP led to a marked reduction in O2 production (Fig. 3). These findings indicate that all four components (membrane, p67PHOX, p47PHOX-P0, and Rac2) were required for activation of the NADPH oxidase, indicating that they are both necessary and sufficient for kinase-dependent cell-free oxidase activation.

Experiments with a cell-free system containing membrane and cytosol indicated that the phosphorylation of p47PHOX was an essential prerequisite for O2 production by the system (31). To see if the same situation prevailed in the recombinant system, the rate of O2 production using p47PHOX-P0 was compared with the rate of O2 production using unphosphorylated p47PHOX. The results (Fig. 4) show that O2 production in this system required the phosphorylation of p47PHOX. It is possible that further phosphorylation of p47PHOX by a membrane-associated kinase might also be necessary for the O2-forming activity in this system. The addition of the protein kinase inhibitor GF-109203X to the reaction mixture, however, had no effect on O2 production, indicating that phosphorylation at least by GF-109203X-inhibitable membrane-associated kinases such as activated protein kinase C was not a factor in these experiments.

The effect of protein concentration on O2 production in the recombinant system was next examined. In these experiments activity was monitored at varying concentrations of the three

Fig. 2. O2 production by GST-p47PHOX-P0-activated leukocyte NADPH oxidase as a function of time. Incubations were carried out as described in the text, using the cytochrome c assay. Components were omitted from the assays as indicated. The results shown are representative of three or more separate experiments. The means ± S.E. for the final points (22 min) are 388 ± 46 (complete), −67 ± 4.2 (no GST-p47PHOX-P0), −4 ± 8 (no p67PHOX), −50 ± 4.1 (no GST-Rac2), and −12.5 ± 4.2 (no recombinant proteins) mol of O2/mol of cytochrome b555/min. Differences between O2 production in the complete assay mixture and O2 production in each of the omission experiments were significant at the level of p < 0.005.

Fig. 3. Requirement for guanine nucleotides in the activation of the NADPH oxidase by p47PHOX-P0 in the recombinant cell-free system. Experiments were conducted as described in the text; Rac2 was loaded with GTPyS or GDPβS as indicated. The results shown represent the mean ± S.E. of four separate experiments.

Fig. 4. NADPH oxidase activation requires phosphorylated p47PHOX in the recombinant cell-free system. The cytochrome c assay was conducted as described in the text, except that GST-p47PHOX-P0 or unphosphorylated GST-p47PHOX was added to the assay mixtures as indicated. The protein kinase inhibitor GF-109203X (5 μM) was also added to some of the incubations. Results are expressed as mean ± S.E. of three or more experiments.
recombinant proteins in the presence of a constant amount of membrane (Fig. 5A) and at varying concentrations of membrane in the presence of a constant amount of recombinant proteins (Fig. 5B). The maximal rate of \( \text{O}_2 \) generation by the p47\text{PHOX}\text{P}_6-activated system was 4.6 ± 0.5 nmol \( \text{O}_2 \)/min/10^7 cell eq of membrane (mean ± S.E., \( n = 6 \), equivalent to 190 mol of \( \text{O}_2 \)/mol of cytochrome \( b_{558} \)/min, a value achieved using 5 × 10^6 cell equivalents of membrane (12 pmol of cytochrome \( b_{558} \)), 105 pmol of GST-Rac2, 75 pmol of p67\text{PHOX}, and 70 pmol of GST-p47\text{PHOX}\text{P}_6 at a low level, something from the cytosol that is missing in the cytosolic cell-free system. When the oxidase is activated, p47\text{PHOX}, p67\text{PHOX}, and Rac2 translocate to the membrane in equimolar quantities (32). Therefore we employed approximately equimolar concentrations of the three recombinant cytosolic components (actual stoichiometry 1.5/1/1 for Rac2/p47\text{PHOX}/p67\text{PHOX}). Altogether, these results indicate that an excess of either membrane or cytosolic components inhibits \( \text{O}_2 \) production in the recombinant cell-free system. Why this same relationship doesn’t prevail in the cytosol-containing system is a mystery.

**Activation of the NADPH Oxidase by SDS Versus Activation by p47\text{PHOX}\text{P}_6—p47\text{PHOX}\text{P}_6 produced similar rates of production of \( \text{O}_2 \) in the recombinant and cytosolic cell-free systems (Fig. 7A). In contrast when SDS was used as the stimulus, the rate of \( \text{O}_2 \) production in the recombinant cell-free system was approximately 50% of the rate seen with cytosol (Fig. 7B), and both rates were considerably greater than the rates obtained in the p47\text{PHOX}\text{P}_6-activated systems. These results strongly suggest that while p47\text{PHOX}\text{P}_6 is sufficient to activate the oxidase at a low level, something from the cytosol that is missing in the recombinant cell-free system is required for maximal activation of the oxidase.**

**DISCUSSION**

It has been known for many years that p47\text{PHOX} becomes heavily phosphorylated on serine residues when the oxidase is activated (8–14, 33). Here we present evidence that p47\text{PHOX} phosphorylated by protein kinase C is capable of activating the leukocyte NADPH oxidase in a recombinant cell-free system consisting of neutrophil membrane, p67\text{PHOX} and Rac2, therefore identifying the minimum cytosolic components necessary for kinase-dependent activation of the oxidase. These findings strongly suggest that the phosphorylation of p47\text{PHOX} that occurs in whole cells during the activation of the leukocyte oxidase is functionally significant and that protein kinase C is a kinase capable of activating p47\text{PHOX} by phosphorylation. In addition, our results with GTP\text{S} and GDP\text{S} confirm that the
activation of the enzyme by protein kinase C also requires the activation of Rac2, as shown previously by others (34, 35). The mechanism of activation of the NADPH oxidase by anionic amphiphiles is still not clear, but our results confirm previous studies demonstrating that the activation of the NADPH oxidase by SDS is not kinase dependent (20). Furthermore, the finding that oxidase activation is associated with the phosphorylation of p47PHOX both in intact cells and in the kinase-dependent cell-free system suggests that as compared with amphiphiles, the activation of the oxidase by protein kinase C may represent a more physiological process. The fact that p47PHOX S379A is nonfunctional both in whole cells and in the kinase-dependent cell-free system, yet is capable of participating in O2\textsuperscript{−} production in the amphiphile-dependent cell-free system (21, 33), further supports the physiological role played by protein kinase C-dependent activation of the oxidase.

The foregoing experiments also showed that the addition of p47PHOX\textsubscript{P}6 to a cell-free oxidase activating system is not enough to activate the oxidase to its full extent. A number of cytosolic components can be postulated as candidate factor(s) that allow the oxidase to become fully activated. These include lipids (e.g. arachidonic acid (36)), proteins (e.g. p47PHOX (37)), possibly other kinases, or perhaps a hitherto undiscovered oxidase component. Nevertheless, our findings show that neutrophil membrane, p47PHOX-p67PHOX and Rac2 are sufficient for protein kinase C-mediated activation of the oxidase, albeit at a relatively low level. The participation of other components in the kinase-dependent activation of NADPH oxidase is the subject of an ongoing investigation.

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Fig. 7. Activation of the NADPH oxidase in the recombinant and cytosolic cell-free system by GST-p47PHOXp6 (A) and SDS (B). The incubations were carried out as described under “Experimental Procedures” using the cytochrome c assay. Results are expressed as mean ± S.E. of three or more experiments.