Phosphorylation of the Respiratory Burst Oxidase Subunit p67phox during Human Neutrophil Activation

REGULATION BY PROTEIN KINASE C-DEPENDENT AND INDEPENDENT PATHWAYS

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The respiratory burst oxidase of phagocytes and B lymphocytes catalyzes the reduction of oxygen to superoxide anion (O$_2^-$) at the expense of NADPH. This multi-component enzyme is dormant in resting cells but is activated on exposure to an appropriate stimulus. The phosphorylation-dependent mechanisms regulating the activation of the respiratory burst oxidase are unclear, particularly the phosphorylation status of the cytosolic component p67phox. In this study, we found that activation of human neutrophils with formyl-methionyl-leucyl-phenylalanine (fMLP), a chemotactic peptide, or phorbol myristate acetate (PMA), a stimulator of protein kinase C (PKC), resulted in the phosphorylation of p67phox. Using an anti-p67phox antibody or an anti-p47phox antibody, we showed that phosphorylated p67phox and p47phox form a complex. Phosphoamino acid analysis of the phosphorylated p67phox revealed only 32P-labeled serine residues. Two-dimensional tryptic peptide mapping analysis showed that phosphorylated p67phox is phosphorylated at the same peptide whether fMLP or PMA is used as a stimulus. In addition, PKC induced the phosphorylation of recombinant GST-p67phox in vitro, at the same peptide as that phosphorylated in intact cells. PMA-induced phosphorylation of p67phox was strongly inhibited by the PKC inhibitor GF109203X. In contrast, fMLP-induced phosphorylation was minimally affected by this PKC inhibitor. Taken together, these results show that p67phox is phosphorylated in human neutrophils by different pathways, one of which involves protein kinase C.

The respiratory burst oxidase, or NADPH$^\dagger$ oxidase, of neutrophils and B lymphocytes is a multicomponent enzyme that catalyzes the NADPH-dependent reduction of oxygen to superoxide anion (O$_2^-$), a precursor of microbicidal oxidants (1, 2). The importance of the oxidase in host defenses is demonstrated by the recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD), a hereditary disorder resulting in defective NADPH oxidase activity (3, 4). Components of this oxidase include cytochrome $b_558$, a membrane-bound flavohemoprotein, the cytosolic proteins p47phox, p67phox, and p40phox, and a small GTP-binding protein Rac2 or Rac1. In resting cells the enzyme is inactive, and its components are distributed between the cytosol and membranes. When cells are activated, the cytosolic components migrate to the membranes and their cytoskeleton fraction, where they associate with cytochrome $b_5$ to form the catalytically active oxidase (5–7).

Activation of neutrophils by formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA) leads to a marked increase in the phosphorylation of multiple proteins on serines, threonines, and tyrosines (8, 9). The functional significance of these phosphorylations and the relevant protein kinases is unclear (2, 10). One of the phosphorylated proteins is the cytosolic oxidase subunit p47phox, which is phosphorylated on several serines (11–14). This phosphorylation is required for the translocation of p47phox to the plasma membrane and for the activation of NADPH oxidase (15, 16). Translocation of p67phox is also essential for the activation of NADPH oxidase, as the oxidase from CGD patients deficient in this protein fails to produce O$_2^-$ (3). While the phosphorylation of p47phox has been extensively investigated, conflicting results have been reported on the phosphorylation of p67phox (17, 18). Using an antibody that specifically immunoprecipitates p67phox, we show here that this protein becomes phosphorylated in human neutrophils stimulated with fMLP or PMA. In addition, we show that the PKC inhibitor GF109203X inhibits PMA-induced phosphorylation of p67phox without affecting fMLP-induced phosphorylation. These results suggest that phosphorylation of p67phox participates in the regulation of NADPH oxidase by PKC-dependent and independent pathways.

EXPERIMENTAL PROCEDURES

**Materials**—PMA, fMLP, phosphatases inhibitors, proteases inhibitors, phosphoserine, phosphothreonine, and phosphotyrosine were from Sigma. Protein kinase C was from Calbiochem or Promega (Madison, WI). GF109203X was from Calbiochem. Sequencing grade trypsin was from Boehringer Mannheim (Germany). SDS-PAGE reagents were from Bio-Rad. [32P]Orthophosphate and [γ-32P]ATP were from DuPont NEN Life Science Products. Rabbit anti-p67phox polyclonal antibody raised against the synthetic peptide extending from amino acid 512 to the C-terminal residue was prepared as described elsewhere (19). The anti-p47phox antibody and EBV-transformed B lymphocytes from p67phox-deficient CGD patient, and normal subjects were kindly provided by Dr. Bernard M. Babior (The Scripps Research Institute, La Jolla, CA).

**Neutrophil Preparation**—Neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrated blood (6). The cells were resuspended at 1 × 10$^6$/ml in phosphate-free buffer (10 mM Hepes, 137 mM NaCl, 5.4 mM KCl, 5.6 mM Na$_2$HPO$_4$, 0.8 mM MgCl$_2$, and 0.025% bovine serum albumin) and treated with 2.5 mM diisopropyl fluorophosphate (DFP) on ice for 20 min.
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**23P Labeling of Neutrophils and Lymphoblasts**—Neutrophils were incubated in phosphate-free buffer containing 1 MCrCl2/10 cells/ml for 1 h at 30 °C. The cells were then washed and activated with PMA (1 μg/ml/10 cells) for 8 min or fMLP (1 μM/10 cells) for 2 min in the presence of 1 mM MgCl2 and 1 mM CaCl2. Activation was terminated with 10 volumes of ice-cold buffer. The cells were pelleted by centrifugation (400 × g for 10 min at 4 °C) and resuspended at 1 × 106 cells/ml in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 15 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1.5 mM phenylmethylsulfonyl fluoride, 1 mM DFP, 0.5% Triton X-100, 25 mM NaF, 5 mM NaVO4, 5 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 0.25 mM sucrose, and 1 mM MnCl2 DNase I), sonicated (3 × 10 s), and centrifuged (100,000 × g, 30 min at 4 °C). Lymphoblasts were labeled with 32P as described previously (16). Briefly, the cells were incubated in phosphate-free medium, then transferred to fresh medium containing 32P (0.2 μCi/ml) and incubated for 4 h at 37 °C. The cells were then activated for 12 min with PMA (1 μg/ml/10 cells) then lysed like the neutrophils.

**Immunoprecipitation Electrophoresis and Immunoblotting Experiments**—Gamma Bind G-Sepharose beads (Pharmacia Biotech Inc.) were equilibrated with lysis buffer containing 1 mg/ml bovine serum albumin at 1 h at 4 °C. The cleared lystate was incubated with p67phox antibody (1/150 dilution) or p47phox antibody (1/200 dilution) or their respective IgG controls in the presence of 50 μl of Sepharose beads overnight at 4 °C with gentle mixing. Then, the beads were washed extensively with lysis buffer without DFP or DNase I. The immunoprecipitated proteins were eluted by boiling in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 0.25% captoethanol). The beads were pelleted by brief centrifugation, and the supernatant was subjected to 10% SDS-PAGE according to Laemmli buffer (62.5 mM Tris-HCl, 10% glycerol, 2.3% SDS, 2% 2-mercaptoethanol). The supernatant was subjected to 10% SDS-PAGE according to Laemmli sample buffer. The proteins were analyzed by SDS-PAGE. The plate was then dried, sprayed with ninhydrin to localize the phosphoamino acid standards, and used for autoradiography.

**RESULTS**

**p67phox Is Phosphorylated in Activated Human Neutrophils**—To determine if p67phox is phosphorylated, neutrophils were loaded with [32P]inorganic phosphate and activated with fMLP or PMA; p67phox was immunoprecipitated with a specific antibody as described under “Experimental Procedures.” Fig. 1A shows the autoradiography of the corresponding gel. While p67phox was weakly phosphorylated in resting cells, its state of phosphorylation clearly increased after stimulation of human neutrophils with PMA (1 μg/ml for 8 min) or fMLP (1 μM for 2 min).

The absence of the phosphorylated protein after immunoprecipitation with the control IgG showed that the presence of this phosphorylated protein was not the result of nonspecific binding to the beads. Corresponding Western blot analysis (Fig. 1B) identified this phosphoprotein as p67phox. Furthermore, as conflicting results have been reported on the phosphorylation of p67phox, possibly due to the use of different and not very specific antibodies, we checked that our antibody did not recognize other proteins than p67phox. As in previous studies (13), the anti-p67phox antibody did not cross-react with another protein around the 67-kDa area. However p47phox, possibly due to the use of different and not very specific antibodies, we checked that our antibody did not recognize other proteins than p67phox. Fig. 2A shows that in EBV-transformed B cells from a CGD patient deficient in p67phox, the anti-p67phox antibody did not cross-react with another protein around the 67-kDa area. However p47phox is normally expressed in these cells and both phosphatase (phagocyte oxidase) proteins are expressed in normal lymphoblasts. In addition, the p47phox-labeled protein was not immunoprecipitated from p67phox-deficient cells but was immunoprecipitated from normal lymphoblasts (Fig. 2, B and C). These results clearly show that p67phox is phosphorylated in activated neutrophils and EBV-transformed B lymphocytes.

**Phosphorilation of p67phox and p47phox Form a Complex**—As in resting cells p47phox and p67phox form a complex, we wondered if these proteins remain in the complex after being phosphorylated. Immunoprecipitation with either p67phox antibody or p47phox antibody resulted in the isolation of both phosphorylated p67phox and p47phox (Fig. 3). However, more of each phosphorylated protein was not the result of nonspecific binding to the beads.
addition, no detectable staining was observed with an anti-phosphotyrosine antibody (data not shown). These results suggest that a Ser/Thr protein kinase, not a tyrosine kinase, phosphorylates p67phox.

**FMLP and PMA Induce the Phosphorylation of p67phox on Serines Located in the Same Peptide—**

The chemotactic peptide fMLP activates neutrophils via a membrane receptor that triggers a multitude of signaling pathways involving phospholipases and protein kinases. However, PMA, which bypasses the receptor, is believed to be more specific for PKC activation. To determine if fMLP and PMA induced the phosphorylation of the same or different phosphopeptides in p67phox, we used two-dimensional tryptic peptide mapping. Fig. 5 shows the presence of one major phosphorylated peptide after neutrophil activation and that the phosphopeptide map of 32P-labeled p67phox from PMA-activated neutrophils was identical to that of labeled p67phox from fMLP-activated neutrophils. In resting cells the same peptide was weakly phosphorylated (data not shown). It is not clear if this peptide contains one or several phosphoserines that could be phosphorylated by different protein kinases.

**PKC Is Involved in p67phox Phosphorylation in Intact Neutrophils—**

The phosphorylation of p67phox is induced by PMA, a direct activator of PKC, suggesting the involvement of this kinase in the phosphorylation of p67phox. To assess this possibility and to determine whether or not PKC is involved in fMLP-induced phosphorylation of p67phox, neutrophils were incubated with the PKC antagonist GF109203X. As seen in Fig. 6, p67phox phosphorylation induced by PMA was strongly inhibited by GF109203X, in contrast, fMLP-induced p67phox phosphorylation was minimally affected by this inhibitor. This suggests that, in addition to the GF109203X-sensitive PKC isoforms, other protein kinases (insensitive to GF109203X) are involved in p67phox phosphorylation.
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PMA or fMLP was inhibited by GF109203X.

Procedures. Data are representative of three experiments.

cipitate with another anti-p67 single band obtained by Western blotting of the immunoprecipitant p67 protein in EBV-transformed B cells. These results strongly support the participation of PKC in p67\textsuperscript{phox} phosphorylation.

The link between respiratory burst oxidase activation and protein phosphorylation is believed to be exclusively mediated by p47\textsuperscript{phox} phosphorylation. Recent and conflicting results (17, 18) have raised the possibility of p67\textsuperscript{phox} phosphorylation. Dusi and Rossi (17) have reported that p67\textsuperscript{phox} is phosphorylated during neutrophil activation, while Heyworth et al. (18) observed no such phosphorylation. The difficulties in p67\textsuperscript{phox} phosphorylation analysis are due mainly to the very low level of the protein in cells (24), the fact that p67\textsuperscript{phox} is very sensitive to proteolysis (25), and the lack of a strongly specific antibody for immunoprecipitation studies. We used an antibody that specifically immunoprecipitates p67\textsuperscript{phox}, as shown by the lack of an immunoprecipitant p67 protein in EBV-transformed B cells from a CGD patient deficient in this protein, as well as the single band obtained by Western blotting of the immunoprecipitate with another anti-p67\textsuperscript{phox} antibody. Using this antibody, we observed that the p67\textsuperscript{phox} component of NADPH oxidase clearly became phosphorylated on stimulation of neutrophils with either fMLP, a receptor-dependent chemotactic activator, or PMA, a PKC activator. The weak phosphorylation observed in nonstimulated cells could correspond to basal phosphorylation or to slight activation during neutrophil isolation.

In resting cells, p47\textsuperscript{phox} and p67\textsuperscript{phox} exist in a complex (22) that translocates to associate with cytochrome b\textsubscript{558} during oxidase activation. In this report we show that p47\textsuperscript{phox} and p67\textsuperscript{phox} remain, at least partially, complexed after their phosphorylation. Partial dissociation cannot be ruled out, as more phosphorylated protein was precipitated by the corresponding specific antibody than by the antibody directed against the other member of the complex. In addition, the observed dissociation could occur naturally or be induced by the antibodies. It has recently been shown that the cytosolic oxidase complex contains, in addition to p67\textsuperscript{phox} and p47\textsuperscript{phox}, a protein named p40\textsuperscript{phox} that participates in oxidase activation (26). We found that p40\textsuperscript{phox} copurified with the phosphorylated p47\textsuperscript{phox}/p67\textsuperscript{phox} complex but was not phosphorylated.

Our results show that p67\textsuperscript{phox} undergoes phosphorylation on serine residues during neutrophil activation, without threonine or tyrosine phosphorylation. These results suggest that a Ser/Thr protein kinase, not a tyrosine kinase, induces the phosphorylation of p67\textsuperscript{phox}. Our observation that GF109203X, a PKC inhibitor, strongly inhibited PMA-induced phosphorylation and barely modified fMLP-induced phosphorylation, points to both PKC-dependent (isoforms sensitive to GF109203X) and PKC-independent (or GF109203X-insensitive) pathways in the phosphorylation of p67\textsuperscript{phox}. Indeed, human neutrophils express in addition to the \& PKC isoforms the PKC\textgreek{i} (27). The PKC inhibitor GF109203X could be more effective against the \& PKC isoforms than the \textgreek{i} one. However, we found that PKC\textgreek{i} is not able to phosphorylate p67\textsuperscript{phox} in vitro. Whether or not GF109203X inhibits PKC\textgreek{i}, this result suggests that PKC\textgreek{i} is not involved in p67\textsuperscript{phox} phosphorylation. Two-dimensional phosphopeptide mapping showed that the same p67\textsuperscript{phox} peptide was phosphorylated after fMLP and PMA stimulation. It is conceivable that this peptide contains several serines that are phosphorylated by different protein kinases: the different sensitivity of p67\textsuperscript{phox} phosphorylation to the PKC inhibitor (GF109203X) when induced by fMLP or PMA supports this hypothesis. Whatever the other kinases involved, the phosphorylation of recombinant GST-p67\textsuperscript{phox} in vitro by purified PKC, on this same peptide, suggests that PKC participates in the phosphorylation of p67\textsuperscript{phox}. After neutrophil activation with PMA or fMLP, p47\textsuperscript{phox} is phosphorylated on several serines (13, 14), the result obtained by tryptic peptide mapping of p67\textsuperscript{phox} suggests that p67\textsuperscript{phox} has less phosphorylated sites than p47\textsuperscript{phox}. However, the phosphorylation of both proteins could have a crucial importance in the regulation of NADPH-oxidase activation.

Several lines of evidence support a role of PKC in NADPH

\begin{itemize}
  \item J. El Benna and M. Gougerot-Pocidalo, unpublished observations.
  \item P M.-C. Dang, M.-A. Gougerot-Pocidalo, and J. El Benna, manuscript in preparation.
\end{itemize}
oxidase activation. PMA, an activator of PKC, is a strong stimulus of $O_2^-$ production in whole cells (28). Purified p47$^{phox}$ is a good substrate for PKC in vitro (29). Staurosporine, a powerful inhibitor of PKC, inhibits superoxide production and p47$^{phox}$ phosphorylation (15, 30). The data presented here provide clear evidence that, in addition to p47$^{phox}$, p67$^{phox}$ itself could play a role in the regulation of NADPH oxidase by phosphorylation/dephosphorylation reactions and that the phosphorylation events involve a PKC-dependent pathway. Little is known of the possible role of other protein kinases in the regulation of NADPH oxidase. It has recently been suggested that cyclic AMP-dependent protein kinase, mitogen-activated protein kinase (14, 31), and p21-activated kinase (32) could regulate NADPH oxidase by phosphorylating p47$^{phox}$. Our findings suggest that protein kinases other than PKC may participate in p67$^{phox}$ phosphorylation. The kinases involved in this process are currently under investigation.

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