The superoxide-generating NADPH oxidase complex of phagocytic cells is a multicomponent system containing a membrane-bound flavocytochrome b and a small G protein Rac as well as cytosolic factors p67phox (phagocyte oxidase), p47phox, and p40phox, which translocate to the membrane upon activation. In a previous paper, we reported that p40phox undergoes phosphorylation on multiple sites upon stimulation of the NADPH oxidase by either phorbol 12-myristate 13-acetate or by formyl peptide with a time course that is strongly correlated with that of superoxide production (Fuchs, A., Bounou, A. P., Rabilloud, T., and Vignais, P. V. (1997) Eur. J. Biochem. 249, 531–539). In this study, through phosphoamino acid and tryptic peptide maps of in vivo and in vitro phosphorylated p40phox, we show that p40phox is phosphorylated on serine and threonine residues during activation of the NADPH oxidase in dimethyl sulfoxide-differentiated HL60 promyelocytes as well as in isolated human neutrophils. In vitro phosphorylation studies using casein kinase II and protein kinase C (PKC) as well as the effect of various protein kinase inhibitors on the isoelectric focusing pattern of p40phox in whole cell lysates point to a role of a PKC type kinase in the phosphorylation of p40phox. Directed mutagenesis of all PKC consensus sites enable us to conclude that Thr^{154} and Ser^{315} in p40phox are phosphorylated during activation of the NADPH oxidase.

In response to invasive microorganisms, neutrophils and other phagocytic cells react violently to produce superoxide anion and other microbicidal toxic oxygen derivatives in the phagocytosis vacuole. This phenomenon is known as the respiratory burst, and the mechanism by which these cells regulate the burst is not yet fully elucidated. The production of superoxide anion is assigned to a multicomponent system, the NADPH oxidase, which consists of a membrane-bound flavocytochrome b and a small G protein Rac as well as cytosolic factors p67phox (phagocyte oxidase), p47phox, and p40phox (for review, see Refs. 2–4). Known mechanisms underlying the activation of the respiratory burst include translocation to the membrane of the cytosolic proteins, specific src homology 3 (SH3)/polyproline motif interactions (5–10), as well as phosphorylation events on p47phox, p67phox, and p40phox (1, 11, 12). Translocation of the three phox proteins is dependent upon the presence of flavocytochrome b in the membrane (13), and the translocation of p40phox appears to be p47phox-dependent and is mediated by p67phox (14). Interestingly, the SH3 domain of p40phox down-regulates activation of the NADPH oxidase (15). As shown by two hybrid studies (6, 7) and by in vitro binding assays (16–18), p47phox interacts with both p40phox and p67phox through its proline-rich COOH-terminal region (6, 18). It seems plausible that some modification occurs during oxidase activation which could probably change the specificity of the proline-rich domain of p47phox for either the SH3 domain of p40phox or the COOH-terminal SH3 domain of p67phox. This modification could be the phosphorylation of p47phox on a cluster of serine residues close to the proline motif in the COOH-terminal region. However, we have recently stressed the fact that the time course of p40phox phosphorylation is strongly correlated with that of superoxide production (1), and we have postulated that p40phox phosphorylation could therefore play a critical role in the rearrangement of the ternary complex consisting of p47phox, p67phox, and p40phox during the respiratory burst. In this report we identify activation-induced phosphorylation sites on p40phox and describe experimental data supporting a critical role of the state of phosphorylation of these residues on the structure of the cytosolic activating complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—[^23]P, at 4,000Ci/mmol was purchased from NEN Life Science Products. Protein A-Sepharose beads, the enhanced chemiluminescence detection kit, and [γ-[^32]P]ATP were from Amersham Pharmacia Biotech. Protein kinase C (PKC) from rat brain was from Boehringer Mannheim. RPMI medium and fetal calf serum were from Life Technologies, Inc. Protein A-horseradish peroxidase conjugate was from Bio-Rad. Polyvinylidene difluoride (PVDF) protein transfer membranes (ProBlott) were from Applied Biosystems. Nitrocellulose membranes were from Schleicher & Schuell. SeeBlue protein molecular weight standards were from Novex. Thrombin was from Sigma, and endoprotease-Lys-C from Boehringer Mannheim.

**Antisera**—Anti-p40phox antisera was raised in rabbits using a synthetic peptide corresponding to the NH₂-terminal 18 amino acid residues of p40phox (1). Anti-p67phox antisera was obtained using purified recombinant p67phox protein expressed in the baculovirus/insect cell system as described (19). After four antigen injection boosts at 4-week intervals, the serum was tested against a total cell lysate by Western blot. All antisera recognized a unique band at the appropriate molecular weight. Specificity was confirmed by testing the preimmune sera in parallel. A COOH-terminal anti-p40phox antisera was a kind gift from

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[^3] The abbreviations used are: phox, phagocyte oxidase; SH3, src homology 3; PKC, protein kinase C; PVDF, polyvinylidene difluoride; PMA, phorbol 12-myristate 13-acetate; GFX, 3-[N-(dimethylamino)propyl-3-indolyl]-4-[3-indolyl]maleimide; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CKII, casein kinase II.

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Prof. Segal (University College, London).

Neutrophil Preparation and Metabolic Labeling—Neutrophils were isolated from buffy coats as described (20). Neutrophils were suspended at 5 x 10\textsuperscript{6} cells/ml in 10 mM HEPES, pH 7.4, containing 157 mM NaCl, 0.8 mM MgCl\textsubscript{2}, 5.4 mM KCl, and 5.6 mM glucose and treated with 5 mM dithiothreitol and 20% glycerol for 30 min at room temperature. The cells were washed once and suspended at 10\textsuperscript{6} cells/ml in the same buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM diethyldithiocarbamate (DDC) and incubated for 90 min at 30 °C. The cell suspension was then supplemented with 1 mM okadaic acid, 0.5 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2} and warmed to 37 °C for 3 min before the addition of 1 μg/ml phorbol 12-myristate 13-acetate (PMA). After 3 min at 37 °C, cells were pelleted by centrifugation and lysed directly in the indicated lysis buffer.

Differentiation of HL60 Cells, Metabolic Labeling, and Activation of the NADPH Oxidase—HL60 cells were subcultured, differentiated in 1.25% dimethyl sulfoxide, and activated as described (1). Cells were washed twice in phosphate-buffered saline consisting of 10 mM HEPES, pH 7.4, 137 mM NaCl, 3 mM KCl and cultured in phosphate-free RPMI medium supplemented with 20 mM glucose, 20 mM HEPES, pH 7.4, 1 mM glutamine, and 0.5 mM cysteine \textsuperscript{35}S. After 3 h at 37 °C, cells were pretreated with 1 mM okadaic acid for 10 min before activation with PMA at a final concentration of 1 μg/ml for 3 min at 37 °C. In PKC inhibition studies, bisindolylmaleimide (GF) was added at a final concentration of 5 μM to the cells 10 min before activation.

Immunoprecipitation Experiments—For immunoprecipitation experiments, antibodies were cross-linked beforehand to protein A-Sepharose beads as described (1) and were stored in NaCUP, with 0.01% NaN\textsubscript{3} at 4 °C until needed.

Immunoprecipitation Assays Using the Anti-p67\textsuperscript{phox} Antiserum—For each immunoprecipitation assay, cells were harvested, metabolically labeled, and then activated as described above. After the activation step, the cell pellets were resuspended in 600 μl of ice-cold Nonidet P-40 lysis buffer consisting of 50 mM HEPES, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol supplemented with 1 mM diisopropyl fluorophosphate, 10 μg/ml leupeptin, 10 mM NaF, 125 mM okadaic acid, 250 μM Na\textsubscript{3}VO\textsubscript{4}, and 1 mM p-nitrophenyl phosphate. After a 15-min incubation on ice, the homogenate was centrifuged for 15 min at 20,000 x g. The supernatant was incubated with 30 μl of micro-crystal pET\textsuperscript{657} antibody-protein A-Sepharose beads for no more than 45 min at 4 °C. Longer incubation times resulted in partial dephosphorylation of p40\textsuperscript{phox}. Even with careful control of incubation temperature and the addition of freshly prepared antiphosphatases, no phosphorylation could be visualized with incubation times longer than 2 h. After four 1-ml washes in Nonidet P-40 buffer, the immunocomplexes were solubilized in Laemmli polymerization buffer and subjected to SDS-PAGE on an acrylamide gel followed by electrotransfer onto a PVDF or nitrocellulose membrane. The membrane was dried and radioactivity detected as above.

In Vitro Proteolytic Digestion—After immunoprecipitation was carried out on metabolically labeled cells, the immunocomplex still linked to the protein A-Sepharose beads was incubated with 2 units of thrombin in 20 μl of ice-cold Nonidet P-40 lysis buffer containing 50 mM HEPES, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol supplemented with 1 mM diisopropyl fluorophosphate, 10 μg/ml leupeptin, 10 mM NaF, 125 mM okadaic acid, 250 μM Na\textsubscript{3}VO\textsubscript{4}, and 1 mM p-nitrophenyl phosphate. After a 15-min incubation on ice, the homogenate was centrifuged for 15 min at 20,000 x g. The supernatant was incubated with 30 μl of micro-crystal pET\textsuperscript{657} antibody-protein A-Sepharose beads for no more than 45 min at 4 °C. Longer incubation times resulted in partial dephosphorylation of p40\textsuperscript{phox}. Even with careful control of incubation temperature and the addition of freshly prepared antiphosphatases, no phosphorylation could be visualized with incubation times longer than 2 h. After four 1-ml washes in Nonidet P-40 buffer, the immunocomplexes were solubilized in Laemmli polymerization buffer and subjected to SDS-PAGE on an acrylamide gel followed by electrotransfer onto a PVDF or nitrocellulose membrane. The membrane was dried and radioactivity detected as above.

Synthesis of Phosphopeptides—Peptides phospho-TRK and phospho-TRK were synthesized using Fmoc (N-[9-fluorenyl]methoxycarbonyl) precursors on an Applied Biosystems 432A peptide synthesizer in J. Garin’s laboratory (Chimie des Protéines, CEA-Grenoble). Cleavage from the resin and removal of side chains were performed by treatment with 90% trifluoroacetic acid, 5% thioanisole, and 5% ethanedithiol.

RESULTS

Phosphoamino Acid Analysis and Tryptic Peptide Mapping of in Vivo Phosphorylated p40\textsuperscript{phox}—We had shown previously that p40\textsuperscript{phox} is phosphorylated during the course of NADPH oxidase activation (1). To identify the amino acids that are modified in tight correlation with the level of superoxide production, we first undertook a phosphoamino acid analysis of in vitro phosphorylated p40\textsuperscript{phox} dimethyl sulfoxide-differentiated HL60 cells were metabolically labeled with \textsuperscript{35}P and activated by PMA. p47\textsuperscript{phox}-p40\textsuperscript{phox} complexes were immunoprecipitated using an anti-p67\textsuperscript{phox} antiserum. The immunoprecipitate was subjected to SDS-PAGE, and the resolved proteins were transferred to a PVDF membrane. p40\textsuperscript{phox} was identified by Western blotting, and the band carrying p40\textsuperscript{phox} was excised from the PVDF membrane. Hydrolysis of peptide bonds was achieved by concentrated hydrochloric acid, and phosphoamino
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The P40phox-p47phox-p67phox complex using the anti-p67phox antiserum, we added thrombin to both the resting complex and the activated complex. The digest was analyzed by Western blot followed by exposure of the blot to a PhosphorImager screen. Panel A in Fig. 3 is the enhanced chemiluminescence image of the blot immunodetected by the NH2-terminal anti-p40phox antiserum. Before digestion, p40phox was present in equal amounts in both the resting complex (lane 1) and the activated complex (lane 2). The resting and activated complexes were then incubated with thrombin (lanes 3 and 4, respectively). An extensive digestion of p40phox is seen in the resting complex (lane 3) with the appearance of the NH2-terminal 16-kDa moiety of p40phox. The presence of the COOH-terminal 23-kDa moiety can also be visualized with a COOH-terminal anti-p40phox antibody (not shown), with a greater amount of the COOH terminus in lane 3 than in lane 4. Interestingly, the effect of thrombin on the activated complex is not as extensive (lane 4). For a large part, in the activated complex p40phox has resisted digestion by thrombin: the NH2-terminal 16-kDa moiety is far less immunodetected, whereas the native 40-kDa protein is more abundant than in the resting complex digest (lane 3). The blot was then exposed to a PhosphorImager screen. Panel B shows the incorporation of 32P, in the resting and activated complexes, before and after digestion with thrombin. Surprisingly, the profile of phosphate incorporation is not modified by thrombin digestion (lane 4 versus lane 2). The NH2-terminal 16-kDa and COOH-terminal 23-kDa products immunodetected with the NH2-terminal and COOH-terminal anti-p40phox antisera carry no labeled phosphate. Thus, only nonphosphorylated p40phox has been digested by thrombin in the resting and activated complexes. This result also explains the extensive digestion of the resting complex versus the activated complex because much more nonphosphorylated p40phox is present in the resting complex than in the activated complex. We also noted that in vitro phosphorylation of rp40phox by PKC protected the recombinant protein from digestion by thrombin (not shown). Thus, protection from proteolytic cleavage by thrombin is brought by the addition of phosphate groups on p40phox alone and does not result from the activation of the other components of the activated complex.

**Tryptic Peptide Mapping and Phosphoamino Acid Analysis of 40phox Phosphorylated in Vitro by CKII and PKC**—We had shown previously that both CKIIa and PKC are able to phosphorylate p40phox in vitro. This is not surprising because p40phox holds consensus sites bearing either threonine or serine for both PKC and CKII. We therefore undertook a tryptic peptide mapping of rp40phox phosphorylated in vitro by either PKC or CKIIa. The aim of this experiment was to assign to the peptide spots from the in vitro phosphorylation map described in a previous section to either a phosphorylation by PKC or by CKIIa, bearing in mind that Thr251, Thr274, and Thr277 are located in consensus sites recognized by both PKC and CKIIa.

The tryptic maps illustrated in Figs. 4A and 5A were obtained after phosphorylation by CKIIa and PKC, respectively. All phosphorylated spots were eluted from the cellulose plates as described in Ref. 24 and subjected to phosphoamino acid analysis. Only serine residues were phosphorylated in vitro by CKIIa, whereas both threonine and serine residues were phosphorylated by PKC (Figs. 4B and 5B). This was confirmed by a direct phosphoamino acid analysis of phosphorylated rp40phox in both cases. By depositing both samples on the same TLC plate, each phosphorylated spot was well individualized, proving that in vitro PKC and CKII do not phosphorylate the same sites on p40phox (data not shown). All of these results point to a likely role of PKC in the in vitro phosphorylation of p40phox during superoxide production.

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2 J. Garin, unpublished observation.
Two-dimensional Analysis of the Effect of a PKC Inhibitor on the Isoelectric Focusing Pattern of p40<sub>phox</sub> in Vivo in Differentiated HL60 Cells as Well as in Isolated Human Neutrophils—To ascertain that PKC is involved in p40<sub>phox</sub> phosphorylation, we studied the effect of a potent and selective inhibitor of PKCs, GFX, on the two-dimensional profile of p40<sub>phox</sub> from PMA-activated differentiated HL60 cells. This study was also carried out with human neutrophils isolated...
from whole blood and activated by PMA. Identical results were obtained with differentiated HL60 cells and human neutrophils. Only the results obtained with neutrophils are presented here. Fig. 6 gives the $^{33}\text{P}$ incorporation profiles of p40$^{phox}$ isolated from the neutrophil lysate by immunoprecipitation. Fig. 6B gives the corresponding Western blot profiles of p40$^{phox}$. In resting neutrophils, p40$^{phox}$ was present mainly as three species characterized by pI values of 6.6, 6.3, and 6.1, as was already shown in resting differentiated HL60 cells (1). Only the 6.1 species had incorporated labeled phosphate. This suggests that either the 6.3 species has a very low phosphorylation turnover or that its more acidic migration results from a post-translational modification of p40$^{phox}$ different from phosphorylation yet to be defined. Upon activation by PMA, two additional spots with more acidic pI values of 5.8 and 5.9 were revealed by radiolabeling (panel A) and immunodetection (panel B). PMA-induced phosphorylation of p40$^{phox}$ was precluded by pretreatment with GFX and concomitantly oxidase activation was abolished (not shown).

The existence of a strong link between the in vivo phosphorylation of p40$^{phox}$ and the activity of a PKC type kinase can be inferred from the following: 1) p40$^{phox}$ is phosphorylated upon the addition of PMA, a potent activator of PKC; 2) the phosphorylation of p40$^{phox}$ is strongly inhibited by GFX; and 3) the tryptic map of p40$^{phox}$ phosphorylated in vitro by PKC is very similar to that obtained after activation-induced in vivo phosphorylation. This very strong correlation prompted us to mutate all consensus PKC sites in p40$^{phox}$ and study the in vitro phosphorylation of these mutants by tryptic peptide mapping.

**In Vitro Analysis of Mutant Forms of p40$^{phox}$—**Threonine residues at positions 154, 211, 251, 274, and Ser 315 were mutagenized into alanine residues to generate the mutants p40-T154A, p40-T211A, p40-T251A, p40-T274A, p40-T315A, and p40-S315A. The directed mutagenesis was performed directly onto the p40$^{phox}$ cDNA cloned in a bacterial expression plasmid. Mutants and wild-type p40$^{phox}$ were expressed as proteins fused to thioredoxin and bearing a polyhistidine tag for easy purification on a nickel affinity column. Fusion proteins were digested with enterokinase to yield the p40$^{phox}$ protein moiety.

With GFX in vitro phosphorylation by PKC in the presence of $\gamma$-$^{32}\text{P}$ATP, proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. A tryptic map was carried out on each phosphorylated band. Tryptic peptide maps of the p40-T154A, p40-S315A, and p40-T154A/S315A mutants were obtained as described above. The nature of the mutation is given under each map. Noteworthy peptide spots are indicated by arrows. Numbers correspond to those of Fig. 5.

**FIG. 6.** Effect of GFX on the isoelectric focusing pattern of immunoprecipitated neutrophil p40$^{phox}$. The cytosolic phox complex was immunoprecipitated from isolated $^{33}\text{P}$-radiolabeled human neutrophils as described under "Experimental Procedures." The immunocomplex was subjected to two-dimensional gel electrophoresis and transferred to a nitrocellulose membrane. $^{33}\text{P}$ incorporation was visualized by a PhosphorImager apparatus (panel A), and p40$^{phox}$ was immunodetected with the NH$_2$-terminal anti-p40$^{phox}$ antiserum (panel B). In panel A is shown the isoelectric focusing pattern of $^{33}\text{P}$-labeled p40$^{phox}$ in the resting state (0), after activation of cells by PMA (PMA), and after treatment of cells with 5 $\mu$M GFX for 10 min before the addition of PMA (GFX + PMA). In panel B is given the corresponding Western blot profiles of p40$^{phox}$. pI values are given at the top of the figure. Two additional acidic species of p40$^{phox}$ formed in activated neutrophils are indicated by arrows.

**FIG. 7.** Tryptic peptide maps of mutant p40$^{phox}$ phosphorylated in vitro by PKC. Recombinant mutant forms of p40$^{phox}$ were expressed and purified as described under "Experimental Procedures." After phosphorylation by PKC, tryptic peptide maps were obtained as described above. The nature of the mutation is given under each map. Noteworthy peptide spots are indicated by arrows. Numbers correspond to those of Fig. 5.
FIG. 8. Identification of the major spots as phospho-TR and phospho-TRK. A tryptic peptide map of wild-type p40<sup>phox</sup> phosphorylated in vitro by PKC was run with 1 μg of the phospho-TR or phospho-TRK synthetic peptide. Ninhydrin staining of the TLC plates is shown underneath the corresponding PhosphorImage.

Two in vivo phosphorylation sites have been identified on p40<sup>phox</sup> as being threonine 154 localized 20 residues upstream of the SH3 domain and serine 315 at the COOH terminus of p40<sup>phox</sup>.

Thr<sub>154</sub> is in a basic region of p40<sup>phox</sup> NH<sub>2</sub>-Arg-Arg-Leu-Arg-Pro-Arg-Thr<sup>154</sup>-Arg-Lys-Val-Lys-COOH which is excessively sensitive to proteolytic cleavage. 7 residues out of the 11 in the above sequence are basic residues. Digestion of p40<sup>phox</sup> by thrombin cuts the protein at arginine 153 immediately adjacent to the major phosphorylation site at threonine 154. Digestion by endoprotease-Lys-C cuts the protein in the same region (not shown). This region is adjacent to the SH3 domain of p40<sup>phox</sup> (residues 175–224). This stretch could therefore act as a very exposed hinge between the NH<sub>2</sub> terminus and the SH3-containing COOH terminus. The presence of phosphate on Thr<sub>154</sub> inhibits the digestion by thrombin at residue Arg<sup>153</sup>, possibly by steric hindrance. The negative charges brought by the phosphate residue could also trigger a change of conformation of p40<sup>phox</sup> which could participate in the transition of the cytosolic phox complex from an inactive to an active state.

The second phosphorylation site identified on p40<sup>phox</sup> is serine 315, localized in the COOH terminus of the protein which has been shown to interact with the inter-SH3 domain of p67<sup>phox</sup> (6, 7). This second site might therefore play a strategic role in the in vivo phosphorylation and superoxide production in differentiated HL60 cells stimulated by PMA or fMet-Leu-Phe-Lys. Here we show that p40<sup>phox</sup> is phosphorylated in an identical manner in human neutrophils stimulated by PMA and that p40<sup>phox</sup> phosphorylation and superoxide production are inhibited by GFX, a potent inhibitor of PKCs. Finally, tryptic peptide mapping of in vivo and in vitro phosphorylated p40<sup>phox</sup> points to a role of a PKC type kinase in the activation-induced phosphorylation of p40<sup>phox</sup>. The tryptic phosphorylation pattern obtained with differentiated HL60 cells stimulated by PMA is identical to the patterns obtained either with isolated neutrophils stimulated by PMA or with differentiated HL60 cells stimulated by formyl peptide at micromolar concentrations (not shown), suggesting that the activation of the signaling pathway through cell surface receptors activates a PKC-type kinase responsible for p40<sup>phox</sup> phosphorylation.

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